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Rola małych, niekodujących cząsteczek
RNA w regulacji rozwoju bakteriofaga
 $\Phi 24_B$ oraz jego gospodarza, bakterii
Escherichia coli produkującej toksynę
Shiga

The role of small, non-coding RNA molecules in
regulating the development of bacteriophage
 $\Phi 24_B$ and its host, the Shiga toxin–producing
bacterium *Escherichia coli*

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N A R O D O W E C E N T R U M N A U K I

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Streszczenie rozprawy doktorskiej

Bakterie *Escherichia coli* produkujące toksynę Shiga, określane jako STEC (ang. Shiga toxin-producing *E. coli*), stanowią wysoce patogenne drobnoustroje odpowiedzialne za ciężkie choroby przewodu pokarmowego oraz zagrażające życiu powikłania ogólnoustrojowe u człowieka. Wirulencja STEC jest bezpośrednio związana z obecnością w ich genomach bakteriofagów Stx przenoszących geny niebezpiecznej dla zdrowia ludzi toksyny Shiga. Fagi Stx należą do rodziny fagów lambdoidalnych i wykazują znaczne podobieństwa w organizacji genomu oraz przebiegu cyklu życiowego do bakteriofaga λ , najlepiej poznanego faga łagodnego. Jednym z najdokładniej scharakteryzowanych przedstawicieli tej grupy wirusów bakteryjnych jest bakteriofag $\Phi 24_B$, stanowiący model badawczy w niniejszej rozprawie doktorskiej.

Podczas infekcji fag łagodny wprowadza do komórki bakteryjnej gospodarza swoje liniowe, dwuniciowe DNA i wybiera jedną z dwóch alternatywnych dróg rozwojowych: lizogeniczną lub lityczną. W trakcie lizogenii genom faga integruje się z chromosomem gospodarza, przyjmując formę profaga i replikuje się pasywnie wraz z genomem bakteryjnym. W tym stadium większość genów fagowych (w tym geny kodujące toksynę Shiga) pozostaje nieaktywna na poziomie transkrypcyjnym, a produkcja toksyny jest skutecznie zahamowana. Indukcja profaga, do której dochodzi pod wpływem działania różnych czynników zewnętrznych, inicjuje cykl lityczny. DNA faga ulega wówczas wycięciu z chromosomu gospodarza, replikuje się niezależnie od genomu bakterii, a następnie ulega transkrypcji i stanowi matrycę do syntezy fagowych białek strukturalnych oraz enzymów litycznych. Proces ten prowadzi ostatecznie do lizy komórki gospodarza i uwolnienia do światła jelita potomnych cząstek fagowych wraz z dużymi ilościami toksyny Shiga. Indukcja profaga może być wywołana przez różnorodne czynniki środowiskowe i fizjologiczne, w tym promieniowanie UV, nadtlenek wodoru oraz niektóre antybiotyki, dlatego nie powinny być one stosowane w leczeniu tego typu infekcji. Samo działanie toksyny Shiga polega na zahamowaniu syntezy białek poprzez zablokowanie translacji na etapie elongacji, co prowadzi do śmierci komórek i uszkodzenia tkanek. Klinicznie, infekcje STEC manifestują się krwawą biegunką,

bólem brzucha oraz wymiotami, a u 15–20% przypadków dochodzi do rozwoju ciężkich powikłań, takich jak krwotoczne zapalenie jelita grubego, zespół hemolityczno–mocznicowy (HUS) lub zakrzepowa plamica małopłytkowa (TTP). Szczególnie podatne na rozwój powikłań są dzieci poniżej piątego roku życia oraz osoby w podeszłym wieku, przy czym odnotowywana w tych grupach wiekowych śmiertelność może sięgać nawet 87%. Obecnie stosowane strategie terapeutyczne skierowane przeciwko bakteriom STEC mają swoje ograniczenia, a antybiotykoterapia jest niezalecana, ponieważ może stymulować indukcję profaga i uwalnianie toksyny, a tym samym nasilać objawy infekcji.

Etap podjęcia decyzji o wyborze rozwoju lizogenicznego lub litycznego u fagów lambdoidalnych jest ściśle regulowany i zależy zarówno od stanu fizjologicznego komórki gospodarza, jak i od czynników regulatorowych kodowanych w genomie faga. Głównym czynnikiem kontrolującym przełączenie pomiędzy cyklami życiowymi faga jest represor CI – białko wiążące DNA, które hamuje transkrypcję z głównych promotorów fagowych (p_L i p_R), zapobiegając jednocześnie ekspresji większości fagowych genów, w tym tych kodujących białka związane z lizą oraz toksynę Shiga. Wejście faga w cykl lityczny wymaga inaktywacji represora CI. Jednym z najlepiej scharakteryzowanych mechanizmów tego procesu jest aktywacja bakteryjnej odpowiedzi SOS w następstwie uszkodzenia DNA, prowadząca do zależnej od RecA autokatalitycznej degradacji zarówno represora LexA gospodarza, jak i fagowego represora CI. Oprócz regulacji zależnej od odpowiedzi SOS, kodowane w genomie faga antyrepresory (w tym Cro, Ant, Roi i D_ant) mogą antagonizować działanie CI poprzez konkurencję o wiązanie w obrębie miejsc operatorowych głównych promotorów fagowych, umożliwiając w ten sposób ekspresję genów, których produkty białkowe odpowiadają za lizę komórki bakteryjnej. Ostatecznie dominacja represorów lub antyrepresorów w komórce determinuje kierunek rozwoju faga.

Oprócz białek regulatorowych, małe, niekodujące RNA (sRNA) okazały się funkcjonować jako istotne modulatory ekspresji genów nie tylko u bakterii, ale i u bakteriofagów. Prokariotyczne cząsteczki sRNA mają zazwyczaj długość od 50 do 550 nukleotydów i regulują ekspresję genów na poziomie potranskrypcyjnym poprzez parowanie się z docelowymi mRNA lub poprzez oddziaływanie z białkami

w celu modulowania ich aktywności. Najlichniesza grupa bakteryjnych sRNA działa poprzez komplementarne wiązanie się z docelowym mRNA. Można tu wyodrębnić dwie podgrupy: sRNA zakodowane w pozycji *cis* oraz *trans*. Geny *cis*-kodowanych cząsteczek sRNA są zlokalizowane w genomie, w tym samym rejonie co ich geny docelowe, ale na nici antysensownej. Cząsteczki te wykazują pełną komplementarność z docelowym transkryptem, co prowadzi do wysoce swoistej regulacji, często poprzez hamowanie translacji lub indukowanie degradacji mRNA. Natomiast geny cząsteczek sRNA zakodowanych w pozycji *trans* są zlokalizowane w innych rejonach genomu niż docelowe miejsca wiązania dla tych cząsteczek i z reguły wiążą się z docelowymi transkryptami w obrębie krótszych sekwencji o niepełnym stopniu komplementarności. Dla stabilnego wiązania często wymagają obecności białek opiekuńczych, takich jak Hfq lub ProQ, oraz zazwyczaj pełnią funkcję globalnych regulatorów, oddziałując na wiele różnych mRNA, których produkty są zaangażowane m.in. w odpowiedź na stres czy proces adaptacji komórki do warunków środowiskowych.

U eukariontów wyodrębnia się klasę wyjątkowo krótkich regulatorowych RNA, tzw. mikroRNA, które podobnie jak prokariotyczne sRNA odgrywają kluczową rolę w regulacji potranskrypcyjnej. Cząsteczki te, zwykle o długości 19–25 nukleotydów, powstają z dłuższych prekursorów i zostały dobrze scharakteryzowane pod względem mechanizmu biogenezy oraz pełnionych funkcji. Co istotne, wirusy infekujące komórki eukariotyczne również posiadają geny kodujące mikroRNA. Cząsteczki te regulują ekspresję zarówno genów samego wirusa, jak i gospodarza, ułatwiając infekcję oraz utrzymanie wirusa w komórce.

Podczas gdy większość dotąd opisanych prokariotycznych sRNA jest dłuższa (powyżej 50 nt) i nie podlega procesowi dojrzewania, najnowsze doniesienia wskazują, że małe RNA podobne do mikroRNA mogą również funkcjonować w systemach bakteryjnych, a ich geny zlokalizowane są zarówno w genomie bakterii, jak i faga. Cząsteczki te stanowią jednak niewielką frakcję opisanych dotąd fagowych i bakteryjnych sRNA, przy czym cząsteczki sRNA fagowego pochodzenia zostały dużo słabiej poznane, niż te zakodowane w genomach bakterii. Cząsteczkom tym poświęciliśmy pracę przeglądową, włączoną do niniejszej rozprawy doktorskiej jako **praca nr 1**. W pracy tej dokonaliśmy szczegółowej analizy

opisanych w literaturze sRNA fagowego pochodzenia, proponując ich podział ze względu na procesy, które kontrolują. W ten sposób zidentyfikowaliśmy sRNA uczestniczące w regulacji m.in.: (i) replikacji DNA faga, (ii) procesu lizogenizacji bakterii, (iii) podziału komórkowego, a także mające wpływ na (iv) metabolizm komórki, (v) jej odpowiedź na stres, a nawet (vi) patogenność [**praca nr 1**].

Wśród opisanych w **pracy nr 1** regulatorowych sRNA wspomniana została również fagowa cząsteczka 24B_1, której odkrycie w 2015 roku stanowiło istotny punkt zwrotny w badaniach nad prokariotycznymi cząsteczkami sRNA, podobnymi do eukariotycznych mikroRNA. 24B_1 to 20-nukleotydowa cząsteczka sRNA powstająca z 80-nukleotydowego transkryptu prekursorowego produkowanego przez niosącego geny toksyny Shiga bakteriofaga $\Phi 24_B$. Cząsteczka ta stanowi pierwsze funkcjonalne sRNA o rozmiarze mikroRNA, które zidentyfikowano u bakteriofagów. Rola, znaczenie i funkcje tej cząsteczki zostały szczegółowo opisane przeze mnie i innych współautorów w **pracy nr 2**. W artykule tym zbadaliśmy wpływ cząsteczki 24B_1 na rozwój faga oraz jego gospodarza, bakterii *E. coli*, w warunkach jej nadekspresji. Dodatkowa kopia genu *24B_1* została wprowadzona do komórek na plazmidzie. W serii eksperymentów analizujących fizjologiczne aspekty rozwoju faga potwierdziliśmy, że pod względem funkcjonalnym cząsteczka 24B_1 stymuluje utrzymanie stanu profaga w komórkach *E. coli*. Zaobserwowaliśmy m.in., że nadekspresja fagowego sRNA 24B_1 z naturalnego promotora (p_{24B_1}) znacząco wpływa na wydajność plonu fagowego. Podczas infekcji z wykorzystaniem dzikiego typu faga $\Phi 24_B$ podwyższony poziom cząsteczki 24B_1 spowodował spadek efektywności produkcji potomnych cząstek fagowych (plonu fagowego) w cyklu litycznym, podczas gdy wydajność adsorpcji faga do powierzchni komórek gospodarza była istotnie zwiększona na wczesnych etapach infekcji. Pomimo zwiększonej wydajności adsorpcji, komórki bakteryjne z nadekspresją 24B_1 wykazywały obniżoną efektywność lizy oraz zwiększoną przeżywalność w porównaniu do szczepów kontrolnych. Efekty te towarzyszyły wyższemu odsetkowi lizogenów wśród komórek, które przetrwały infekcję, co wskazuje, że zwiększony poziom 24B_1 w komórce stymuluje rozwój faga w kierunku lizogenii. Tendencja ta była obserwowana przy różnych wartościach m.o.i. (ang. multiplicity of infection) oraz w warunkach sprzyjających zarówno

lizogenii, jak i rozwojowi litycznemu. Chociaż przeżywalność bakterii była niższa w warunkach bogatych w składniki odżywcze, sprzyjających cyklowi litycznemu, to jednak lizogeny stanowiły większość komórek, które przetrwały infekcję.

Zgodnie z wynikami eksperymentów fizjologicznych, dodatkowo przeprowadzone analizy z wykorzystaniem RT-qPCR wykazały, że nadekspresja cząsteczki 24B_1 prowadzi do zmian w ekspresji genów fagowych. Podczas gdy poziomy ekspresji badanych genów we wczesnych etapach infekcji były początkowo podobne w komórkach bakteryjnych z naturalnym i podwyższonym poziomem produkcji 24B_1, to jednak geny związane z lizogenią, takie jak *cl*, *cII* i *cIII*, wykazywały znacznie wyższy poziom ekspresji w późniejszych etapach infekcji, w wariancie z nadekspresją 24B_1. Geny te kodują kluczowe białka regulatorowe ścieżki lizogenicznej, co potwierdza wcześniejsze obserwacje, że 24B_1 sprzyja ustanowieniu lizogenii.

Oprócz wpływu na rozwój faga i ekspresję jego genów, nadekspresja cząsteczki 24B_1 spowodowała również zmiany w metabolizmie komórki gospodarza, przyczyniając się do przeprogramowania szlaków energetycznych komórki bakteryjnej na bardziej sprzyjające utrzymaniu stanu lizogenii. Podwyższony poziom 24B_1 spowodował istotne obniżenie wewnątrzkomórkowego stężenia ATP, nie wpływając przy tym na tempo wzrostu bakterii ani przeżywalność komórek. Z kolei analiza metabolomu w warunkach nadekspresji 24B_1 dodatkowo wykazała zaburzenia w centralnym metabolizmie węgla, charakteryzujące się obniżeniem poziomu fumaranu i akumulacją NAD^+ , kluczowych metabolitów z cyklu kwasu cytrynowego. Zastanawiając się, z czego może wynikać spadek poziomu fumaranu w komórce, analizie poddaliśmy sekwencje genów kodujących podjednostki enzymu dehydrogenazy bursztynianowej Sdh, który jest odpowiedzialny za przemianę bursztynianu w fumaran. Wykonana analiza bioinformatyczna pozwoliła zidentyfikować potencjalne miejsce wiązania dla 24B_1 w regionie 5' mRNA genu *sdhB*. Badania wiązania RNA *in vitro* za pomocą metody różnicowej migracji kompleksów w żelu poliakrylamidowym (ang. mobility gelshift assay) wykazały, że dojrzała cząsteczka 24B_1, w przeciwieństwie do swojego prekursora, jest zdolna do wiązania się z tym fragmentem mRNA, chociaż oddziaływanie to było stosunkowo słabe, co sugeruje,

że dodatkowe czynniki lub specyficzne warunki panujące w komórce mogą być wymagane do efektywnego wiązania *in vivo*. Wiązanie to, choć słabe, może wskazywać na bezpośrednie działanie 24B_1 na ekspresję genu *sdhB*, co może skutkować obniżeniem poziomu enzymu w komórce oraz zaburzeniem cyklu Krebsa na etapie przemiany bursztynianu w fumaran. W efekcie prowadzi to do nagromadzenia się NAD⁺ (albowiem nie ulega przemianie do NADH) oraz do spadku poziomu ATP w komórce wskutek zaburzenia całego procesu przemian energetycznych. Przypuszcza się, że niski poziom ATP w komórce może upośledzać działanie proteaz zależnych od ATP, co sprawia, że represory cyklu litycznego pozostają aktywne i kierują faga na drogę rozwoju lizogenicznego.

Analizy strukturalne cząsteczek wykazały ponadto, że dojrzała cząsteczka 24B_1 posiada mniej rozbudowaną strukturę drugorzędową niż jej prekursor, co zwiększa jej dostępność do oddziaływań międzycząsteczkowych *in vivo*. Z drugiej strony jednak, dalsze badania wykazały, że białko opiekuńcze Hfq wiąże formę prekursorową 24B_1, natomiast nie oddziałuje z dojrzałą cząsteczką, co sugeruje rolę Hfq w stabilizacji prekursora lub w procesie biogenezy, a nie na poziomie działania dojrzałego sRNA.

Podsumowując najważniejsze osiągnięcia z **pracy nr 2**, uzyskane wyniki wskazują, że 24B_1 działa jako regulatorowe RNA kontrolujące zarówno metabolizm komórki gospodarza, jak i ekspresję genów fagowych, celem utrzymania faga w rozwoju lizogenicznym.

Dalsze badania genomu faga Φ24_B oraz analizy transkryptomiczne doprowadziły do identyfikacji kolejnego, fagowego, bardzo małego RNA – cząsteczki UpRoi1 o wielkości zaledwie 30 nukleotydów. W **pracy nr 3** poddaliśmy tę cząsteczkę szczegółowej charakterystyce i wykazaliśmy, że w przeciwieństwie do 24B_1, cząsteczka UpRoi1 stymuluje lityczny rozwój faga. Zidentyfikowaliśmy potencjalne miejsca wiązania dla tej cząsteczki zarówno w genomie faga, jak i bakterii oraz eksperymentalnie potwierdziliśmy, że UpRoi1 bezpośrednio oddziałuje zarówno z transkryptem fagowym, jak i bakteryjnym, a wiązania te są stabilizowane przez białko opiekuńcze ProQ.

Cząsteczka UpRoi1 jest kodowana przez gen *uproi1*, który znajduje się w rejonie regulatorowym genomu faga pomiędzy genami *ant* i *roi*, ale na nici

przeciwnej oraz posiada zidentyfikowane sekwencje promotora p_N i terminatora T2. Analizy ilościowe z wykorzystaniem metody RT-qPCR potwierdziły powstawanie transkryptu UpRoi1 w lizogennej komórce i wykazały, że jego poziom wzrasta znacząco po indukcji profaga, przy czym najwyższa ekspresja obserwowana była podczas transkrypcji z naturalnego promotora p_N . Nawet w warunkach spontanicznej indukcji, wariant z nadekspresją UpRoi1 wykazywał znacząco podwyższony poziom tego transkryptu w komórce, potwierdzając jego efektywną produkcję pomimo braku egzogenego induktora. Dalsze eksperymenty wykazały, że nadekspresja cząsteczki UpRoi1 istotnie wpłynęła na obniżenie przeżywalności bakterii po infekcji i nasilała lizę komórek w hodowli zarówno w warunkach spontanicznej, jak i wymuszonej mitomycyną C indukcji profaga. Efekt ten towarzyszył około dziesięciokrotnemu wzrostowi liczby cząstek fagowych oraz zmniejszeniu procentowego udziału żywych komórek gospodarza w hodowli, co potwierdzają wyniki analiz z wykorzystaniem cytometrii przepływowej.

Lokalizacja w genomie faga oraz wysoki poziom komplementarności do potencjalnych sekwencji docelowych sugerowały, że UpRoi1 może działać jako cząsteczka RNA typu *cis*, oddziałując na niepodlegający translacji region 5'UTR transkryptu genu *roi*. Analiza wiązania RNA *in vitro* za pomocą metody różnicowej migracji kompleksów w żelu poliakrylamidowym wykazała bezpośrednie wiązanie UpRoi1 z 100-nukleotydowym fragmentem 5'UTR mRNA genu *roi*. Białko opiekuńcze ProQ zostało zidentyfikowane jako stabilizujące ten kompleks, natomiast białko Hfq nie wykazało powinowactwa do tego kompleksu. Analizy spektroskopowe również potwierdziły tę interakcję. Obserwowane zmiany wartości absorbancji w zakresie promieniowania UV oraz zmiany w widmie dichroizmu kołowego (CD) wskazują na reorganizację strukturalną w wyniku tworzenia kompleksu. Uzyskana wartość stałej wiązania ($K_{bind} = 3,81 \times 10^6 \text{ M}^{-1}$) wskazuje na wysoki stopień powinowactwa, a ujemna wartość zmiany energii swobodnej Gibbsa ($\Delta G = -37,52 \text{ kJ/mol}$) świadczy o spontanicznym, termodynamicznie korzystnym i stabilnym charakterze tego wiązania. Analiza widma CD dodatkowo wykazała, że chociaż UpRoi1 samodzielnie charakteryzuje się ograniczoną stabilnością strukturalną, jego interakcja z 5'UTR mRNA genu *roi* sprzyja powstawaniu wysoko uporządkowanej konformacji bogatej w struktury helikalne.

Dodatkowo potwierdzone przez nas wiązanie regulatora odpowiedzi SOS, białka LexA, do regionu promotora naturalnego p_N sugeruje, że ekspresja UpRoi1 jest zintegrowana z systemem odpowiedzi SOS u gospodarza. Z kolei analizy RNA-seq i RT-qPCR wykazały zwiększoną ekspresję fagowych genów antyrepresorowych (*cro*, *roi*, *ant* i *d_ant*) w komórkach z nadekspresją UpRoi1, podczas gdy poziom transkryptu represora CI pozostawał niezmienny, co wskazuje, że regulacja sprzyja aktywności antyrepresorów i nie wpływa bezpośrednio na poziom mRNA genu *ci* w komórce.

Na tej podstawie zaproponowaliśmy zależny od cząsteczki UpRoi1 mechanizm przełączenia cyklu życiowego faga $\Phi 24_B$ [Ryc. 9 z pracy nr 3]. W regulacji tej białko LexA, pełniące rolę represora w bakteryjnym systemie odpowiedzi SOS, wiąże się z sekwencją promotorową p_N genu *uproi1* i hamuje jego transkrypcję. W wyniku uszkodzeń DNA (powstałych na skutek np. działania światła UV lub innych czynników) w komórce gromadzą się fragmenty ssDNA, których obecność z kolei prowadzi do aktywacji białka naprawczego RecA. Aktywacja RecA inicjuje jednocześnie dwa mechanizmy inaktywacji białka CI (odpowiedzialnego za utrzymanie stanu lizogenii): bezpośrednią autoproteolizę białka CI oraz pośrednią, będącą efektem degradacji białka LexA, do której dochodzi właśnie w wyniku aktywności białka RecA. Degradacja represora LexA prowadzi do odblokowania transkrypcji genu *uproi1*. Produkowana jest wówczas cząsteczka UpRoi1, która wiąże się z 5'UTR genu antyrepresora Roi, a dokładnie w obrębie sekwencji terminatora T1 zlokalizowanej powyżej genu *roi* i pokrywającej się z jego potencjalną sekwencją promotorową p_{946} . Wydaje się, że ko-lokalizacja terminatora T1 oraz promotora stanowi zabezpieczenie przed przedwczesną transkrypcją genu *roi*. Dopiero obecność cząsteczki sRNA UpRoi1 i jej interakcja z sekwencją 5'UTR mRNA genu *roi* prawdopodobnie destabilizuje strukturę terminatora T1, umożliwiając w ten sposób zwiększoną ekspresję genu *roi* z promotora p_{946} . Dochodzi wówczas do produkcji antyrepresora Roi, którego produkt może konkurować z CI o wiązanie do miejsc operatorowych głównych promotorów fagowych p_R i p_L . W efekcie oba mechanizmy, bezpośredni i pośredni, prowadzą do zniesienia hamującego działania CI; dochodzi do transkrypcji wczesnych genów fagowych znajdujących się

pod kontrolą promotrów p_R oraz p_L i następuje przełączenie cyklu życiowego faga na rozwój lityczny.

W **pracy nr 3** dowiedliśmy również, że poza regulacją ekspresji genów fagowych, UpRoi1 moduluje również ekspresję genów gospodarza znajdujących się w pozycji *trans* do genu cząsteczki. Analiza transkryptomiczna wykazała obecność 158 genów z podwyższonym poziomem ekspresji oraz 83 genów z obniżonym poziomem ekspresji w komórkach z nadekspresją cząsteczki UpRoi1. Szczególnie zauważalne było znaczące podwyższenie poziomu ekspresji genów (*cheA*, *cheW*, *tar*, *tap*, *tsr*, *motA*, *motB*, *fliC* i *flgL*) zaangażowanych w ruchy chemotaktyczne i składanie wici. Profilowanie proteomiczne wykazało podwyższony poziom białek związanych z aktywnością transkrypcyjną i translacyjną, przy jednoczesnym istotnym obniżeniu poziomu DnaA, inicjatora replikacji DNA chromosomalnego. Sugeruje to możliwą redystrybucję zasobów komórkowych gospodarza na rzecz procesu replikacji genomu fagowego podczas rozwoju litycznego.

Dalsze badania z wykorzystaniem narzędzi bioinformatycznych wraz z analizą powyższych danych transkryptomicznych pozwoliły na zidentyfikowanie mRNA genu *flgL* jako bezpośredniego celu bakteryjnego dla cząsteczki UpRoi1. Gen ten koduje białko łączące hak i włókno w strukturze wici bakteryjnej. Przeprowadzony test różnicowej migracji kompleksów w żelu poliakrylamidowym wykazał, że RNA UpRoi1 i 85-nukleotydowy fragment mRNA *flgL* tworzą wiązanie o wysokim powinowactwie, które może być stabilizowane przez białko opiekuńcze ProQ. Pomiar spektroskopowe potwierdziły silną i spontaniczną interakcję ($K_{\text{bind}} = 4,71 \times 10^7 \text{ M}^{-1}$; $\Delta G = -43,75 \text{ kJ/mol}$). W kontekście fizjologicznym nadekspresja UpRoi1 zwiększała ruchliwość bakterii, co potwierdzono obserwacją wzmożonego rozproszenia komórek w agarze, w warunkach spontanicznej indukcji, skorelowaną ze wzrostem poziomu ekspresji *flgL*. Sugerujemy, że taka pozytywna, zależna od UpRoi1 regulacja może wynikać z preferencji faga do rozwoju litycznego w warunkach bogatych w składniki odżywcze i potrzeby ukierunkowania lizogennej komórki na poszukiwania takiego środowiska.

W efekcie badań nad cząsteczkami sRNA fagowego pochodzenia, wspólnie z naukowcami z Wydziału Chemii Uniwersytetu Gdańskiego oraz Wydziału Biologii Uniwersytetu Adama Mickiewicza w Poznaniu, wypracowaliśmy jeszcze jedno

ważne, interdyscyplinarne rozwiązanie [praca nr 4]. Jest to procedura analizy wiązania małych cząsteczek sRNA z docelowymi transkryptami, łącząca technikę badania wiązania RNA *in vitro* za pomocą metody różnicowej migracji kompleksów w żelu poliakrylamidowym z chemicznymi analizami spektroskopowymi opartymi na obserwacji zmian absorbancji w zakresie promieniowania UV oraz zmian w widmie CD. Połączenie trzech technik pozwoliło wypracować wiarygodne narzędzie do badania siły, stabilności i spontaniczności (samorzutności) procesu wiązania sRNA–mRNA.

W świetle uzyskanych dotąd wyników, możemy wskazać na przeciwstawne działanie cząsteczek 24B_1 i UpRoi1 oraz wysunąć główny wniosek, że fagowe sRNA o wielkości eukariotycznych mikroRNA mogą pełnić funkcję czynników regulatorowych kontrolujących przełączanie się cyklu życiowego faga. Podobne funkcje pełnią cząsteczki mikroRNA wirusów infekujących komórki eukariotyczne, np. herpeswirusów, które wykazują istotne podobieństwa w cyklu życiowym z łagodnymi bakteriofagami ogonkowymi, w tym fagami Stx. Obie te grupy wirusów wykazują zdolność do przełączania się pomiędzy utajonym stanem latencji/lizogenii a rozwojem litycznym. W okresie latencji herpeswirusów (lizogenii u fagów) większość genów wirusowych pozostaje nieaktywna, podczas gdy ograniczony zestaw genów aktywnych zapewnia utrzymanie genomu w komórce i unikanie mechanizmów obronnych gospodarza. Reaktywacja wirusa tudzież indukcja profaga uruchamia wycięcie, a następnie replikację fagowego DNA oraz jego dalszy rozwój lityczny, który prowadzi do zniszczenia komórki gospodarza i uwolnienia potomnych wirusów. U herpeswirusów mikroRNA odgrywają kluczową rolę w regulacji przełączenia pomiędzy cyklami życiowymi wirusa. Większość opisanych cząsteczek kontroluje fazę latencji poprzez hamowanie ekspresji genów litycznych, ale nieliczne sprzyjają również rozwojowi litycznemu. Te wirusowe mikroRNA często są kodowane na nici antysensownej i regulują ekspresję zarówno genów wirusowych, jak i genów gospodarza zaangażowanych m.in. w proces apoptozy, metabolizm, czy migrację komórek.

Opisane powyżej wyniki niewątpliwie wskazują, że fagowe sRNA typu mikroRNA stanowią dodatkowy mechanizm regulacji ekspresji genów i kontrolują rozwój bakteriofagów oraz interakcje fag–gospodarz. Poprzez modulowanie

ekspresji genów fagowych represorów, antyrepresorów oraz genów gospodarza, te małe RNA przyczyniają się do precyzyjnej kontroli cyklu życiowego faga. Zrozumienie mechanizmów działania fagowych sRNA nie tylko pogłębia podstawową wiedzę na temat biologii fagów, ale także otwiera nowe perspektywy dla strategii terapeutycznych mających na celu ograniczenie produkcji toksyny Shiga poprzez stabilizację stanu utajonego fagów Stx w patogennych szczepach *E. coli*. W świetle tych wyników można również wskazać, że fagi Stx niosą geny cząsteczek sRNA funkcjonalnie analogiczne do eukariotycznych mikroRNA. Sprzyjająca lizogenii cząsteczka 24B_1 stanowi pierwszy dowód na istnienie mechanizmu regulacyjnego podobnego do tego funkcjonującego u wirusów infekujących komórki eukariotyczne. Następstwem tego odkrycia było zidentyfikowanie i scharakteryzowanie kolejnego fagowego sRNA, UpRoi1, które również odgrywa kluczową rolę w podejmowaniu przez faga decyzji o wyborze ścieżki rozwojowej. Poza rolą w regulacji ekspresji genów fagowych i stymulowaniu rozwoju litycznego, cząsteczka UpRoi1 kontroluje również ekspresję genów gospodarza poprzez bezpośrednie wiązanie się z mRNA genu *flgL*. Oddziaływanie to może ułatwiać rozprzestrzenianie się cząstek wirusowych poprzez stymulację migracji komórek bakteryjnych w kierunku korzystniejszego środowiska, tym samym zapewniając równowagę pomiędzy produkcją cząstek fagowych a przetrwaniem populacji gospodarza. UpRoi1, podobnie jak mikroRNA herpeswirusów, które zwiększają migrację i inwazję komórek gospodarza, wydaje się sprzyjać rozprzestrzenianiu się wirusa poprzez kontrolę procesów życiowych bakterii.

Te obserwacje niewątpliwie potwierdzają hipotezę, że bakteriofagi łagodne niosą geny kodujące regulatorowe sRNA, pod wieloma względami przypominające cząsteczki mikroRNA funkcjonujące w komórkach eukariotycznych. Fagowe sRNA wyłaniają się zatem jako kluczowe regulatory interakcji fag–gospodarz, aktywowane przez sygnały środowiskowe, zmiany stanu metabolicznego komórki oraz zmiany w wielkości populacji bakterii celem ukierunkowania faga na właściwą ścieżkę rozwoju. Interwencja terapeutyczna zorientowana na tego typu cząsteczki sRNA lub ich interakcje z docelowymi transkryptami może stanowić nową strategię kontroli indukcji profaga i ograniczenia produkcji toksyny Shiga podczas infekcji STEC.

Lista publikacji wchodzących w skład rozprawy doktorskiej:

Praca nr 1

Lewandowska, N., Bloch, S., Łukasiak, A., Wesółowski, W., Węgrzyn, G., & Nejman-Faleńczyk, B. (2025). The role of bacteriophage-derived small RNA molecules in bacterial and phage interactions. *Viruses*, 17(6), 834. (<https://doi.org/10.3390/v17060834>).

Praca nr 2

Bloch, S., **Lewandowska, N.**, Zwolenkiewicz, J., Mach, P., Łukasiak, A., Olejniczak, M., Donaldson, L. W., Węgrzyn, G., & Nejman-Faleńczyk, B. (2023). Bacteriophage-encoded 24B_1 molecule resembles herpesviral microRNAs and plays a crucial role in the development of both the virus and its host. *Plos One*, 18(12), e0296038. (<https://doi.org/10.1371/journal.pone.0296038>).

Praca nr 3

Bloch, S., **Lewandowska, N.**, Zwolenkiewicz-Nowak, J., Mazur, M., Chylewska, A., Dąbrowska, A. M., Musiał, N., Czaplewska, P., Wesółowski, W., Łukasiak, A., Ferenc, M., Pietrzycki, M., Węglińska, E., Mach, P., Węgrzyn, G., Olejniczak, M., & Nejman-Faleńczyk, B. (2026). UpRoi1 - the phage-derived microRNA-size non-coding RNA molecule regulating both phage and its bacterial host development. *Biochimica et Biophysica Acta. Gene Regulatory Mechanisms*, 1869(1), 195135. (<https://doi.org/10.1016/j.bbagr.2026.195135>).

Praca nr 4

Lewandowska, N., Bloch, S., Chylewska, A., Dąbrowska, A. M., Mazur, M., Zwolenkiewicz, J., Wesółowski, W., Łukasiak, A., Węglińska, E., Olejniczak, M., Węgrzyn, G., & Nejman-Faleńczyk, B. (2026). Application of ultraviolet (UV) and circular dichroism (CD) spectroscopies to study RNA-RNA interactions of regulatory RNAs of bacteriophage origin. *Methods in Molecular Biology* (Clifton, N.J.), 3004, 123–136. (https://doi.org/10.1007/978-1-0716-5084-4_8).

Abstract of doctoral dissertation

Shiga toxin–producing *Escherichia coli* (STEC) are highly pathogenic bacteria responsible for severe gastrointestinal diseases and life-threatening complications in humans. The virulence of STEC is directly associated with the presence in their genomes of Stx bacteriophages, which carry the genes encoding the Shiga toxin, a dangerous threat to human health. Stx phages belong to the family of lambdoid phages and exhibit significant similarities in genome organization and life cycle to bacteriophage λ , which is the best characterized temperate phage. One of the most studied representatives of this group of bacterial viruses is bacteriophage $\Phi 24_B$, which serves as the model system in this doctoral dissertation.

During infection, a temperate phage injects its linear double–stranded DNA into the bacterial host cell and chooses one of the two alternative developmental pathways: the lysogenic or the lytic cycle. During lysogeny, the phage genome integrates into the host chromosome, maintaining in the form of a prophage and replicating passively along with the bacterial genome. At this stage, most phage genes (including those encoding Shiga toxin) remain transcriptionally inactive, and toxin production is effectively suppressed. Prophage induction, triggered by various external factors, initiates the lytic cycle. At this point, phage DNA is excised from the host chromosome, replicates independently apart from the bacterial genome, and is next transcribed, serving as a template for the synthesis of structural phage proteins and lytic enzymes. This process ultimately leads to lysis of the host cell and the release of progeny phage particles, along with large amounts of Shiga toxin, into the intestinal lumen. Prophage induction may be triggered by a variety of environmental and physiological factors, including UV radiation, hydrogen peroxide, and certain antibiotics, therefore, they should not be used in the treatment of these infections. The mechanism of Shiga toxin action involves inhibition of protein synthesis through blockade of translation at the elongation stage, leading to cell death and tissue damage. Clinically, STEC infections manifest as bloody diarrhea, abdominal pain, and vomiting, and in 15–20% of cases, they can develop to severe complications such as hemorrhagic colitis, hemolytic uremic syndrome (HUS), or thrombotic thrombocytopenic purpura (TTP). Children under five years of age and

elderly individuals are particularly sensitive to these complications, with reported mortality rates in these groups reaching even 87%. Currently, available therapeutic strategies targeting STEC bacteria have significant limitations, and antibiotic therapy is not recommended, as it may stimulate prophage induction and toxin release, thereby worsening the infection.

The choice between lysogenic and lytic development in lambdoid phages is tightly regulated and depends both on the physiological state of the host cell and on regulatory factors encoded within the phage genome. The main factor controlling the switch between phage life cycles is the CI repressor – a DNA-binding protein that inhibits transcription from the major phage promoters (p_L and p_R), thereby preventing the expression of most phage genes, including those encoding lysis-associated proteins and Shiga toxin. The entrance of the phage into the lytic cycle requires inactivation of the CI repressor. One of the best characterized mechanisms of this process is activation of the bacterial SOS response following DNA damage. This leads to RecA-dependent autocatalytic cleavage of both the host LexA repressor and the phage CI repressor. In addition to SOS-dependent regulation, antirepressors encoded in the phage genome (such as Cro, Ant, Roi, and D_ant) can antagonize CI activity by competition for binding at operator sites within the major phage promoters, thereby enabling the expression of genes whose protein products are responsible for bacterial cell lysis. Ultimately, the dominance of repressors or antirepressors within the cell determines the developmental pathway of the phage.

In addition to regulatory proteins, small non-coding RNAs (sRNAs) have emerged as important controllers of gene expression not only in bacteria but also in bacteriophages. Prokaryotic sRNAs are typically 50 to 550 nucleotides in length and regulate gene expression at the posttranscriptional level, either by base pairing with target mRNAs or by interacting with proteins to modulate their activity. The largest group of bacterial sRNAs functions through complementary binding to target mRNAs. Within this group, two subtypes can be distinguished: *cis*-encoded and *trans*-encoded sRNAs. Genes encoding *cis*-acting sRNAs are located in the genome in the same region as their target genes but on the opposite strand. These molecules pair to target RNA with full complementarity that results in highly specific regulation, often through inhibition of translation or induction of mRNA degradation.

On the other hand, genes encoding *trans*-acting sRNAs, are located in genomic regions distinct from their target binding sites. Besides, these molecules typically pair through shorter sequences with limited complementarity. For stable interaction, they often require the support of RNA chaperone proteins such as Hfq or ProQ. These sRNAs generally act as global regulators, influencing multiple mRNA targets whose gene products are involved, for example, in stress responses and cellular adaptation to environmental conditions.

In eukaryotes, a distinct class of very short regulatory RNAs, known as microRNAs (miRNAs), has been identified. Similar to prokaryotic sRNAs, these molecules play a key role in posttranscriptional regulation. Typically, only 19–25 nucleotides in length, microRNAs are produced from longer precursor transcripts and have been extensively characterized in terms of both their biogenesis and functional roles. Importantly, viruses that infect eukaryotic cells also encode microRNAs in their genomes. These molecules may regulate the expression of both viral and host genes, thereby facilitating infection and promoting viral stability within the host cell.

While most prokaryotic sRNAs that have been described till now are relatively long (above 50 nucleotides) and do not undergo a maturation process, recent reports indicate that microRNA-type small RNAs may also function in bacterial systems, and their genes might be located in both bacterial and phage genomes. Importantly, these very short molecules constitute only a small fraction of the sRNAs identified so far in bacteria and bacteriophages. Moreover, phage-encoded sRNAs remain less well characterized than those encoded in bacterial genomes, so these molecules were reviewed in this doctoral dissertation in the frame of **article no. 1**. In this paper, we performed a detailed analysis of phage-derived sRNAs described in the literature and proposed their classification based on the biological processes they regulate. This approach enabled the identification of sRNAs involved in the regulation of, among others: (i) phage DNA replication, (ii) bacterial lysogenization, (iii) cell division, as well as sRNAs influencing (iv) cellular metabolism, (v) stress response, and even (vi) pathogenicity [**article no. 1**].

Among the regulatory sRNAs described in **article no. 1**, the phage-derived molecule 24B_1 was also mentioned. Its discovery in 2015 set a significant turning

point in studies of prokaryotic sRNAs resembling eukaryotic microRNAs. 24B_1 is a 20-nucleotide sRNA molecule originating from an 80-nucleotide precursor transcript produced by the Shiga toxin-encoding bacteriophage Φ 24_B. This molecule represents the first functional sRNA of microRNA-like size identified in bacteriophages. Its role, significance, and functions were widely characterized by us in **article no. 2**. In this study, we investigated the impact of 24B_1 on the development of the phage and its host, *E. coli*, under conditions of its overexpression. An additional copy of the 24B_1 gene was incorporated into cells on a plasmid. Through a series of experiments analyzing physiological aspects of phage development, we demonstrated that, functionally, 24B_1 promotes maintenance of the prophage state in *E. coli* cells. Notably, overexpression of the phage-encoded sRNA 24B_1 from its natural promoter (p_{24B_1}) significantly affected phage progeny. During infection with wild-type Φ 24_B, elevated levels of 24B_1 sRNA resulted in reduced efficiency of progeny phage production in the lytic cycle, while phage adsorption to the host cell surface was significantly increased at early stages of infection. Despite increased adsorption efficiency, bacterial cells overexpressing 24B_1 exhibited reduced lysis efficiency and increased survival compared to control strains. These effects were coupled with a higher proportion of lysogens among cells that survived infection, indicating that an increased level of 24B_1 in the cell promotes phage development toward lysogeny. This trend was observed across different multiplicities of infection (m.o.i.) and under conditions that favored both lysogenic and lytic development. Although bacterial survival rate was lower under nutrient-rich conditions favoring the lytic cycle, nevertheless, lysogens constituted the majority of cells that survived infection.

Consistent with the results of the physiological experiments, additional RT-qPCR analyses demonstrated that overexpression of the 24B_1 molecule leads to changes in phage gene expression. While the expression levels of the analyzed genes at early stages of infection were initially comparable between bacterial cells with normal and elevated 24B_1 production efficiency, in the overexpression variant, at later stages of infection, genes associated with lysogeny – such as *ci*, *cII*, and *cIII* – showed markedly higher expression levels. These genes encode key

regulatory proteins of the lysogenic pathway, thereby confirming earlier observations that 24B_1 promotes the establishment of lysogeny.

In addition to its impact on phage development and gene expression, overexpression of the 24B_1 molecule altered host cell metabolism, contributing to the reprogramming of bacterial energy pathways toward conditions that are more favorable for maintenance of the lysogenic state. Elevated levels of 24B_1 resulted in a significant reduction in intracellular ATP concentration, with no effect on bacterial growth rate or cell viability. Metabolomic analysis under conditions of 24B_1 overexpression further revealed disruptions in central carbon metabolism, characterized by decreased levels of fumarate and accumulation of NAD^+ , key metabolites of the tricarboxylic acid cycle. To investigate the observed decrease in intracellular fumarate levels, we analyzed the sequences of genes encoding subunits of succinate dehydrogenase Sdh, the enzyme responsible for the conversion of succinate to fumarate. Bioinformatic analysis identified a potential 24B_1 binding site within the 5' region of the *sdhB* gene mRNA. *In vitro* RNA-binding assays, based on differential mobility of molecules and their complexes in a gel, demonstrated that the mature 24B_1 molecule, contrary to its precursor, is capable of binding to this mRNA fragment, although the interaction was relatively weak. This suggests that maybe additional factors or specific intracellular conditions may be required for efficient binding *in vivo*. Despite its low affinity, this interaction may indicate a direct role of 24B_1 in regulating *sdhB* expression, potentially leading to reduced levels of the enzyme and disruption of the Krebs cycle at the point of succinate to fumarate conversion. As a consequence, NAD^+ accumulates (due to its reduced conversion to NADH), and cellular ATP levels decrease as a result of impaired energy metabolism. It is hypothesized that reduced intracellular ATP levels may disturb the activity of ATP-dependent proteases, thereby allowing repressors of the lytic cycle to remain active and directing the phage toward the lysogenic development pathway.

Further structural analyses demonstrated that the mature 24B_1 molecule shows a less complex secondary structure than its precursor, which likely increases its accessibility for intermolecular interactions *in vivo*. On the other hand, the following studies revealed that the RNA chaperone protein Hfq binds the precursor

form of 24B_1, but does not interact with the mature molecule. This observation suggests that Hfq plays a role in precursor stabilization or in the biogenesis process, rather than in the functional activity of the mature sRNA itself.

In summary, the key findings of **article no. 2** indicate that 24B_1 functions as a regulatory RNA that controls both host cell metabolism and phage gene expression, thereby promoting maintenance of the phage in the lysogenic developmental pathway.

Further investigation of the $\Phi 24_B$ phage genome, combined with transcriptomic analyses, led to the identification of another phage-encoded, very small RNA molecule – UpRoi1, containing only 30 nucleotides in length. In **article no. 3**, we performed a detailed characterization of this molecule and demonstrated that, in contrast to 24B_1, UpRoi1 promotes the lytic development of the phage. We identified potential binding sites for this molecule in both the phage and bacterial genomes and experimentally confirmed that UpRoi1 directly interacts with both phage and bacterial transcripts. These interactions are stabilized by the RNA chaperone protein ProQ.

The UpRoi1 molecule is encoded by the *uproi1* gene, located within the regulatory region of the phage genome between the *ant* and *roi* genes, but on the opposite strand, and contains identified p_N promoter and T2 terminator sequences. Quantitative analyses using RT-qPCR confirmed the production of the UpRoi1 transcript in lysogenic cells and demonstrated that its levels increase significantly after prophage induction, with the highest expression observed during transcription from its natural p_N promoter. Even under conditions of spontaneous induction, the UpRoi1 overexpression variant exhibited a significantly elevated level of this transcript in the cell, confirming its efficient production in the absence of the exogenous inducer. Further experiments showed that overexpression of UpRoi1 significantly reduced bacterial survival after infection and enhanced cell lysis in culture under both spontaneous conditions and mitomycin C-induced prophage activation. This effect was coupled with an approximately tenfold increase in the number of phage progeny particles, as well as a decrease in the percentage of viable host cells in the culture, as confirmed by flow cytometry analyses.

The genomic localization of UpRoi1, together with its high level of complementarity to the potential target sequence, suggested that it may function as a *cis*-acting RNA molecule, interacting with the non-translated 5'UTR region of the *roi* gene transcript. *In vitro* RNA-binding analysis demonstrated direct binding of UpRoi1 to a 100 nucleotide long fragment of the 5'UTR of *roi* gene mRNA. The RNA chaperone protein ProQ was identified as a stabilizing factor for this complex, whereas Hfq showed no affinity to it. Spectroscopic analyses further confirmed this interaction. Observed changes in UV absorbance, as well as alterations in circular dichroism (CD) spectra, indicated structural reorganization upon complex formation. The calculated binding constant ($K_{bind} = 3.81 \times 10^6 \text{ M}^{-1}$) indicates high binding affinity, while the negative Gibbs free energy ($\Delta G = -37.52 \text{ kJ/mol}$) reflects a spontaneous, thermodynamically favorable, and stable interaction. CD spectral analysis additionally revealed that although UpRoi1 alone exhibits limited structural stability, its interaction with the structured 5'UTR of *roi* mRNA promotes the formation of a highly ordered conformation enriched in helical structures.

Furthermore, confirmed by us experimentally, the binding of the SOS response regulator, the LexA protein, to the natural p_N promoter region suggests that UpRoi1 expression is integrated with the host SOS response system. In turn, RNA-seq and RT-qPCR analyses revealed increased expression of phage antirepressor genes (*cro*, *roi*, *ant*, and *d_ant*) in cells that overexpressed UpRoi1, while the transcript level of the CI repressor remained unchanged. This indicates that the regulatory mechanism is based on antirepressor activity, instead of the direct effect on the *cl* gene mRNA level in the cell.

Based on these findings, we proposed an UpRoi1-dependent mechanism of the bacteriophage $\Phi 24_B$ life cycle switch [Fig. 9 in the article no. 3]. In this regulatory model, LexA protein, acting as a repressor in the bacterial SOS response system, binds to the p_N promoter sequence of the *uproi1* gene and inhibits its transcription. In response to DNA damage (for example, caused by UV radiation or other stress factors), single-stranded DNA (ssDNA) accumulates in the cell, leading to activation of the DNA repair protein RecA. RecA activation simultaneously initiates two mechanisms of CI inactivation (the repressor responsible for maintaining lysogeny): direct autocleavage of the CI protein and an indirect pathway

resulting from LexA degradation, which is also mediated by RecA activity. Degradation of the LexA repressor relieves transcriptional repression of the *uproi1* gene. As a consequence of this, the UpRoi1 molecule is produced and binds to the 5'UTR mRNA of the *roi* antirepressor gene, specifically within the T1 terminator sequence that is located upstream of the *roi* coding sequence and overlaps with its putative promoter p_{946} . The co-localization of the T1 terminator and p_{946} promoter likely serves as a safeguard against premature transcription of the *roi* gene. In the presence of UpRoi1, whose interaction with the 5'UTR mRNA of the *roi* gene likely destabilizes the T1 terminator structure, the increased expression of *roi* from the p_{946} promoter can occur. This results in the production of the Roi antirepressor, which can compete with CI for binding to operator sites of the major phage promoters p_R and p_L . Finally, both direct and indirect mechanisms lead to the abolition of CI-mediated repression, enabling transcription of early phage genes located under the control of the p_R and p_L promoters and triggering the switch of the phage life cycle toward lytic development.

In **article no. 3**, we also demonstrated that, in addition to regulating phage gene expression, UpRoi1 modulates the expression of host genes located in *trans* position relative to its encoding locus. Transcriptomic analysis revealed 158 genes with increased expression level and 83 genes with decreased expression level in cells overexpressing UpRoi1. Notably, there was a marked upregulation of genes (*cheA*, *cheW*, *tar*, *tap*, *tsr*, *motA*, *motB*, *fliC*, and *flgL*) involved in chemotaxis and flagellar assembly. Proteomic profiling further showed elevated levels of proteins associated with transcriptional and translational activity, alongside a significant decrease in the level of DnaA, the initiator of chromosomal DNA replication. This suggests a potential redistribution of host cellular resources in favor of phage genome replication during the lytic cycle.

Further bioinformatic analyses, combined with the transcriptomic data, enabled the identification of *flgL* mRNA as a direct bacterial target of UpRoi1. This gene encodes a hook filament junction protein essential for flagellar structure. Mobility gelshift binding assay demonstrated that UpRoi1 RNA and an 85-nucleotide-long fragment of *flgL* mRNA formed a high-affinity complex, stabilized by the RNA chaperone protein ProQ. Spectroscopic measurements confirmed a strong

and spontaneous interaction ($K_{\text{bind}} = 4.71 \times 10^7 \text{ M}^{-1}$; $\Delta G = -43.75 \text{ kJ/mol}$). From a physiological perspective, UpRoi1 overexpression increased bacterial motility, as shown by increased dispersion of cells in agar under conditions of spontaneous induction, which correlated with elevated level of *flgL* expression. We propose that this positive, UpRoi1-dependent regulation may reflect a phage strategy favoring lytic development in nutrient-rich environments, potentially directing lysogenic host cells toward conditions conducive to such growth.

As a result of our studies on phage-derived sRNA molecules, conducted in collaboration with researchers from the Faculty of Chemistry at the University of Gdańsk and the Faculty of Biology at Adam Mickiewicz University in Poznań, we developed an additional important interdisciplinary approach [**article no. 4**]. This approach includes a procedure for analyzing the binding of small sRNA molecules to their target transcripts, combining *in vitro* RNA-binding assays based on electrophoretic mobility shift analysis with chemical spectroscopic analyses, including measurements of changes in UV absorbance and circular dichroism spectra. The integration of these three techniques enabled the development of a potent and reliable tool for estimating the strength, stability, and spontaneity (thermodynamic favorability) of sRNA-mRNA interactions.

In light of the results obtained so far, we can point to the opposing activities of the 24B_1 and UpRoi1 molecules and draw the main conclusion that phage-encoded sRNAs of sizes comparable to eukaryotic microRNAs can function as regulatory factors controlling the switch of the phage life cycle. Similar roles are played by microRNAs of viruses infecting eukaryotic cells, such as herpesviruses, which exhibit notable similarities in their life cycles to temperate tailed bacteriophages, including Stx phages. Both groups of these viruses are capable of switching between a latent state (latency in herpesviruses, lysogeny in phages) and lytic development. During latency (or lysogeny), the majority of viral genes remain inactive, while a limited number of expressed genes ensure maintenance of the viral genome integrated into the host cell, and avoidance of host defense mechanisms. Viral reactivation, or prophage induction, initiates excision followed by replication of viral DNA and entry into the lytic cycle, finally leading to host cell lysis and release of progeny virions. In herpesviruses, microRNAs play a crucial role in regulating the

switch between life cycles. Most characterized microRNAs promote latency by repressing the expression of lytic genes, although some also facilitate lytic development. These viral microRNAs are often encoded on the antisense strand and regulate the expression of both viral and host genes involved in processes such as apoptosis, metabolism, and cell migration.

The results described above clearly indicate that microRNA-like phage-encoded sRNAs form an additional mechanism of gene expression regulation and control bacteriophage development and phage-host interactions. By modulating the expression of phage repressors, antirepressors, and host genes, these small RNAs contribute to the precise regulation of the phage life cycle. Understanding the mechanisms of action of phage-encoded sRNAs not only expands fundamental knowledge of phage biology but also opens new perspectives for therapeutic strategies aimed at reducing Shiga toxin production by maintaining the latent state of Stx phages in pathogenic *E. coli* strains. In light of these findings, it can be concluded that Stx phages encode sRNA molecules functionally analogous to eukaryotic microRNAs. The lysogeny-promoting molecule 24B_1 represents the first evidence of a regulatory mechanism resembling that observed in viruses infecting eukaryotic cells. This discovery was further extended by the identification and characterization of another phage-encoded sRNA, UpRoi1, which also plays a key role in phage developmental switch. Beyond its role in regulating phage gene expression and promoting lytic development, UpRoi1 also controls host gene expression through direct binding to the mRNA of the *flgL* gene. This interaction may facilitate the dissemination of viral particles by stimulating bacterial cell motility towards more favorable environments, thereby ensuring a balance between phage production and host population survival. Similar to herpesvirus microRNAs that enhance host cell migration and invasiveness, UpRoi1 appears to control viral spreading through the regulation of essential bacterial life processes.

These observations strongly support the hypothesis that temperate bacteriophages carry genes encoding regulatory sRNAs that, in many aspects, resemble microRNA molecules functioning in eukaryotic cells. Phage-encoded sRNAs emerge here as key regulators of phage-host interactions, activated by environmental signals, triggers, and changes in cellular metabolic state or

fluctuations in bacterial population density, that direct the phage toward the appropriate development pathway. Therapeutic intervention involving such sRNAs, or their interactions with target transcripts, may represent a novel strategy for controlling prophage induction and limiting Shiga toxin production during STEC infections.

List of publications included in the doctoral dissertation:

Article no. 1

Lewandowska, N., Bloch, S., Łukasiak, A., Wesółowski, W., Węgrzyn, G., & Nejman-Faleńczyk, B. (2025). The role of bacteriophage-derived small RNA molecules in bacterial and phage interactions. *Viruses*, 17(6), 834. (<https://doi.org/10.3390/v17060834>).

Article no. 2

Bloch, S., **Lewandowska, N.**, Zwolenkiewicz, J., Mach, P., Łukasiak, A., Olejniczak, M., Donaldson, L. W., Węgrzyn, G., & Nejman-Faleńczyk, B. (2023). Bacteriophage-encoded 24B_1 molecule resembles herpesviral microRNAs and plays a crucial role in the development of both the virus and its host. *Plos One*, 18(12), e0296038. (<https://doi.org/10.1371/journal.pone.0296038>).

Article no. 3

Bloch, S., **Lewandowska, N.**, Zwolenkiewicz-Nowak, J., Mazur, M., Chylewska, A., Dąbrowska, A. M., Musiał, N., Czaplewska, P., Wesółowski, W., Łukasiak, A., Ferenc, M., Pietrzycki, M., Węglińska, E., Mach, P., Węgrzyn, G., Olejniczak, M., & Nejman-Faleńczyk, B. (2026). UpRoi1 - the phage-derived microRNA-size non-coding RNA molecule regulating both phage and its bacterial host development. *Biochimica et Biophysica Acta. Gene Regulatory Mechanisms*, 1869(1), 195135. (<https://doi.org/10.1016/j.bbagr.2026.195135>).

Article no. 4

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Praca nr 1

The role of bacteriophage-derived small RNA molecules in bacterial and phage interactions

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Review

The Role of Bacteriophage-Derived Small RNA Molecules in Bacterial and Phage Interactions

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Abstract: Small regulatory RNAs (sRNAs) play a critical role in bacterial gene expression, modulating various cellular processes, including stress responses, metabolism, virulence, and many others. While well-characterized in bacterial systems, an emerging class of phage-derived sRNAs has been identified, suggesting an underexplored regulatory network at phage–host interactions. These sRNAs, encoded within phage genomes, influence both bacterial and viral life cycles by modulating transcriptional and post-transcriptional gene expression processes. The interplay between phage-derived sRNAs and the host genome reveals a complex network of gene regulation, with an impact on bacterial fitness, pathogenesis, and horizontal gene transfer. This review explores the diverse functions of phage-encoded sRNAs, highlighting recent discoveries and their impact on bacterial physiology and phage–host interactions.

Keywords: bacteriophage sRNAs; small regulatory RNAs; bacteriophages; microRNA



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1. Introduction

Small regulatory RNAs (sRNAs) are essential components involved in post-transcriptional gene regulation and perform diverse functions in cellular processes [1]. In prokaryotes, these non-coding molecules typically range from 50 to 550 nucleotides in length. Their mechanisms of action include interacting with target mRNAs to influence their stability or translation efficiency, and binding to protein targets, thereby modifying the function of the bound protein. The largest group of sRNAs in prokaryotes operates through complementary base pairing with target mRNAs. Within this group, two types of molecules can be distinguished: *cis*-encoded and *trans*-encoded sRNAs (Figure 1). These two types differ in the genomic location of their coding sequences relative to their target genes [2].

Cis-encoded sRNAs are transcribed from the DNA strand antisense to the coding strand of the target transcript and are located within the same genomic region as their target mRNAs [2]. These sRNA molecules are characterized by long, perfect, or near-perfect complementary sequences that enable precise pairing with the target mRNAs (Figure 1A). Some authors call these molecules antisense-encoded sRNAs (asRNAs) and use this name interchangeably with *cis*-encoded sRNAs [3]. In prokaryotic organisms, asRNAs lead to the inhibition of translation or promote mRNA degradation [2]. In this work, we keep the original terms used by the authors to refer to the described sRNA molecules. In contrast,

trans-encoded sRNAs are encoded in genomic regions distinct from their target mRNAs. They contain shorter and imperfect complementary sequences with their target mRNAs, which is why they often require chaperone proteins such as Hfq for efficient binding and stabilization (Figure 1B) [2]. *Trans*-encoded sRNAs typically do not exhibit strict one-to-one specificity. Instead, due to their short and imperfect base-pairing regions, they are capable of targeting multiple mRNA targets that share partial sequence complementarity. *Trans*-encoded sRNAs represent the most abundant class of regulatory small RNAs in prokaryotes and play roles strongly correlated to bacterial stress responses. These sRNAs act as global regulators, mediating cellular adaptation to a variety of environmental stresses, including metabolite and nutrient stress, temperature stress, envelope/outer membrane protein (OMP) stress, iron deficiency, oxidative stress, pH fluctuations, and oxygen stress or anaerobic conditions [4]. Such responses affect gene expression and induce changes in bacterial physiology and/or behavior, linking these responses to numerous other cellular processes [2].

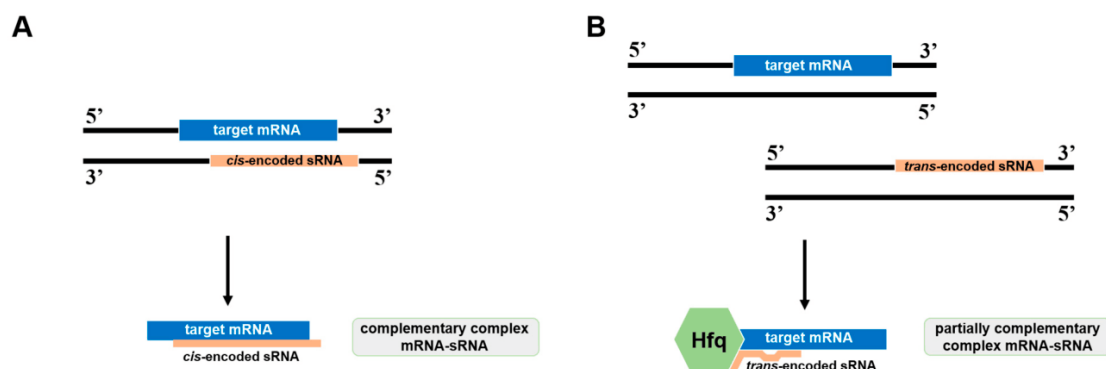


Figure 1. Difference in the genomic location of *cis*-encoded (A) and *trans*-encoded (B) sRNAs to their target gene. Orange boxes show the sRNA, while blue boxes show the target mRNA. Hfq protein is marked in green.

In the 1990s, exceptionally short RNAs were identified in *Caenorhabditis elegans* and were shown to be key regulators of developmental transitions [5]. This discovery brought attention to a previously unappreciated class of sRNAs, known now as microRNAs. Found primarily in eukaryotic organisms, microRNAs are involved in post-transcriptional gene regulation and are characterized by their very short length, typically ranging from 19 to 25 nucleotides [6]. In eukaryotic cells, microRNAs are well-studied and well-defined models, in contrast to their counterparts in prokaryotic organisms [7]. Short, microRNA-size molecules have been reported in prokaryotes; however, their significant biological role remains unexplored [8]. In eukaryotic cells, microRNAs can also originate from viruses that infect them. Similarly, the first functional microRNA-size molecule, named 24B_1, derived from Shiga toxin-converting bacteriophage (shortly Stx phage) Φ 24B was identified a decade ago [9].

The expression of bacteriophage genes is regulated by various mechanisms, including the activity of sRNAs of phage origin. It was demonstrated that phage-derived sRNAs and prophage-carried sRNAs' genes found within bacteriophage-derived regions of bacterial genomes can not only regulate their own gene expression but also influence the gene expression of their bacterial hosts [9,10]. A similar mechanism has been observed in eukaryotic systems, where virus-derived sRNAs can modulate host gene expression, facilitating viral infection [11–13]. This functional similarity between eukaryotic and prokaryotic virus-derived sRNAs suggests a conserved strategy by which viral elements influence host cellular processes.

While phage-originated sRNAs are being increasingly identified, their functions remain largely unknown and unexplored. In our previous reviews, we listed reports about phage-derived sRNAs published until that time [14,15]. In this paper, we enrich the list with new reports on identified phage-derived sRNAs and aim to explore the topic of phage-originated sRNAs, with a particular focus on their role in physiological processes within the cell. Additionally, in a separate table, we have collected 38 sRNAs without a described function (Supplementary Table S1).

2. Phage Replication and Lysogeny Control

While some phages (called virulent) can develop solely by multiplication in bacterial host cells, temperate bacteriophages can undergo two distinct development cycles, lytic and lysogenic. In the lytic cycle, phages hijack the bacterial machinery to produce new virions, leading to host cell lysis; while in lysogeny, the phage genome integrates into the bacterial chromosome or (rarely) occurs in the form of a plasmid, remaining unseen to bacteria until certain conditions trigger phage induction. The switch between these states is tightly regulated by complex genetic and molecular mechanisms, including sRNA of phage origin influence [9,10,14,16–20].

An example of phage sRNA that modulates host processes is VpdS, encoded by the lysogenic phage VP882 [16]. This sRNA plays a crucial role in promoting phage replication in *Vibrio cholerae* and functions in the quorum sensing (QS) responsive regulatory network. The VP882 phage can detect the bacterial QS molecule DPO, which triggers the switch from lysogenic to lytic development. VpdS binds to the major RNA chaperone protein Hfq, influencing both phage and host mRNAs' levels in favor of phage replication. Specifically, it was shown to repress host-encoded sRNAs and compete with them for Hfq binding. This dynamic enables VpdS to shift the regulatory landscape to benefit phage development. Additionally, the study demonstrated that host-encoded sRNAs can antagonize phage replication by targeting phage mRNAs, suggesting a bidirectional RNA-mediated regulatory conflict between host and phage [16].

Another example of such sRNAs is PreS (LPR1), whose gene is located in λ phage genome between *ea10* and *ral* genes, and which is strongly dependent on Hfq binding [17]. PreS plays a crucial role in regulating essential bacterial genes during phage infection, serving as an adaptive strategy for λ phage to enhance its survival and replication [17]. By targeting the host *dnaN* gene, which is critical for DNA replication, PreS optimizes the cellular environment for efficient phage propagation. Notably, *dnaN* expression is upregulated under stress conditions, such as UV irradiation and exposure to alkylating agents, suggesting a potential link between PreS regulation and the bacterial SOS response. The SOS response is crucial in prophage induction as it triggers the phage developmental switch to the lytic cycle. In that study [17], two additional Hfq-dependent sRNAs of phage origin were identified—LPR2 and 6S; however, their functions were not explored (Supplementary Table S1). Nevertheless, the authors of that work speculated that those sRNAs are likely to be associated with the lytic cycle [17].

RNA 24B_1 is a small non-coding phage sRNA that plays a crucial role in maintaining the prophage state and reprogramming host metabolism. It was identified in *Escherichia coli* after induction of the Stx phage Φ 24_B with mitomycin C [9,10]. Similar to herpesvirus microRNAs, 24B_1 is a short 20 nt molecule processed from a longer 80 nt precursor, with Hfq facilitating its maturation [9,10]. Thus, 24B_1 is a microRNA-like molecule operating in prokaryotic cells. It was reported as the first functional prokaryotic microRNA-size molecule, indicating that microRNAs may be unrestricted to eukaryotic organisms [9,10]. Overproduction of 24B_1 promotes the lysogenic developmental pathway of phage Φ 24_B by enhancing the expression of its genes *cI*, *cII*, and *cIII*, which are essential for prophage

maintenance [10]. The 24B_1 microRNA-size molecule is proposed to function as a negative regulator of the *d_ant* gene, which encodes a putative antirepressor protein [9,10]. By repressing *d_ant* expression, 24B_1 supports the activity of the *cI* repressor, thereby maintaining the repression of lytic promoters and promoting lysogeny. In the absence of 24B_1, increased *d_ant* expression leads to the inhibition of the *cI* repressor, resulting in enhanced expression of phage lytic genes. This shift favors more efficient lytic development, impaired lysogenization, quicker prophage induction, and the formation of partially defective virions due to the unbalanced expression of structural phage proteins [9].

An independent study identified an asRNA encoded by the phage PAK_P4, referred to as PAK_P4as15 [18]. Interestingly, such an sRNA is not encoded in the genome of a related phage PAK_P3. This asRNA is associated with the endolysin gene, and it may act as a translational repressor, potentially delaying the lysis of the host cell [18]. Four other sRNAs (PAK_P4as06, PAK_P4as07, PAK_P4as14, and PAK_P4as16) have been identified in this phage, but their functions have not been determined [18].

The small *sar* asRNA plays a crucial role in the regulation of gene expression in bacteriophage P22, particularly in the control of antirepressor synthesis [19]. The key function of *sar* asRNA is repression of the antirepressor by base pairing with the *ant* mRNA, which encodes the antirepressor protein. This base pairing interferes with the initiation of translation of the antirepressor, effectively reducing its synthesis at a post-transcriptional level. Despite the presence of other repressors of *ant* gene expression, *sar* asRNA is essential for the proper regulation of antirepressor synthesis, which is critical for the establishment of lysogeny in P22. The presence of *sar* asRNA ensures optimal antirepressor levels, maintaining the proper balance of gene expression throughout the phage cycles [19].

OOP RNA is a small regulatory RNA encoded by lambdoid bacteriophages. It is transcribed from the *pO* promoter and was initially believed to play a dual role in both the expression of the *cI* gene and as a primer for DNA replication [15]. However, later studies clarified that while OOP RNA does not serve as a primer for DNA synthesis, the *pO* promoter remains important for regulating DNA replication. One of the key functions of the OOP RNA is its role as an asRNA for the *cII* gene, which encodes a crucial regulator of phage development, named *cII*. By binding to *cII* mRNA, OOP RNA inhibits its expression and thereby influences whether the phage undergoes lytic or lysogenic development [15]. Additionally, OOP RNA expression can suppress the Rex exclusion phenomenon, which typically prevents the development of certain bacteriophages that infect lysogenic bacteria. On the transcriptional level, OOP RNA promotes the degradation of *cII* mRNA through RNase III activity, and its overexpression tends to favor the lytic cycle of bacteriophage λ over lysogeny. Recent research has also uncovered additional small RNAs encoded by lambdoid bacteriophages, suggesting that the regulatory roles of RNAs like OOP RNA are even more complex and significant than previously understood [15]. Overall, OOP RNA plays a critical role in regulating gene expression of phage origin and shaping the developmental pathways of bacterial viruses [15].

The aQ RNA is an asRNA produced from the *paQ* promoter during the development of phage λ and plays a key role in the regulation of gene expression [20]. Specifically, aQ RNA downregulates the expression of the *Q* gene, whose product is essential for the activation of late genes during the phage's lytic development. aQ asRNA is proposed to act *in cis* to regulate *Q* gene expression. Additionally, aQ RNA appears to function alongside other promoters, such as *pRE* and *pI*, to coordinate the lysogenic growth response, suggesting its involvement in a broader regulatory network. By controlling *Q* gene expression, aQ RNA critically influences the decision between lysogenic and lytic cycles, thereby shaping the phage developmental pathway [20]. Overall, aQ RNA exemplifies the sophisticated

regulatory strategies employed by phages to fine-tune gene expression and balance their life cycles [20].

3. Bacterial Cell Division and Morphology

Phage sRNAs enable viruses to manipulate host physiology to optimize infection success or persistence, often by targeting key regulatory pathways [14,21–25]. One such example is DicF sRNA from the Qin prophage. This small molecule regulates growth, cell division, and metabolic functions, and influences adaptation to environmental conditions of *E. coli* bacteria [21–25]. DicF (53 nt) is transcribed from the *dicB* operon and functions as a *trans*-acting inhibitor of cell division, particularly under stress conditions such as elevated temperatures [21,23–25]. This sRNA inhibits bacterial cell division by directly base-pairing with *ftsZ* mRNA, preventing translation and blocking the synthesis of FtsZ, a bacterial tubulin homolog, essential for cytokinesis [21,22,24,25]. In addition to affecting *ftsZ*, DicF also regulates metabolic genes, and its production can be toxic to *E. coli* [21]. It has been shown that DicF represses *xylR* and *pykA* mRNAs, which are involved in xylose metabolism and pyruvate kinase activity, respectively [21]. The influence of DicF on bacterial morphology is particularly clear under anaerobic conditions, where it promotes cell filamentation by suppressing FtsZ production [21,22,25]. This regulation is stabilized by the interaction of DicF with enolase, a component of the RNA degradation system, which allows the sRNA to accumulate and exert its inhibitory effect. The resulting filamentous growth pattern is especially relevant in pathogenic strains of *E. coli*, where multiple copies of DicF facilitate morphological adaptations in oxygen-limited environments [25]. The Hfq protein further increases DicF stability, protecting it from degradation and enhancing its ability to inhibit FtsZ [21,25]. This regulatory mechanism enables *E. coli* to adjust its morphology in response to fluctuating oxygen levels, highlighting DicF's role in bacterial stress adaptation. The inhibitory effects of DicF extend beyond *ftsZ* repression, as its secondary structure resembles a Rho-independent transcription terminator, suggesting that it may modulate RNA stability and degradation of division-related transcripts [21,25].

In summary, as an untranslated product of the *dicB* operon, DicF exemplifies the sophisticated post-transcriptional regulation empowered by small RNAs to fine-tune bacterial cell cycle control. In addition to its role in cell division, DicF also regulates metabolic processes.

4. Superinfection Exclusion

Superinfection exclusion (SIE) is a strategy employed by bacteriophages to prevent further infections of the same host cell by other phages. This is achieved through various mechanisms, such as modifying bacterial surface receptors to block phage adsorption or inhibiting the injection of phage genetic material into the host cell. Although SIE provides short-term benefits to phages by monopolizing host cells, it may cause long-term costs, such as reduced burst size [26].

STnc6030 is a phage-derived asRNA transcribed from the BTP1 prophage genome and has been proposed to regulate gene expression by base-pairing with complementary mRNA sequences. Specifically, STnc6030 interacts with the mRNA of the BTP1 tailspike gene, reducing transcript stability and inhibiting translation [27]. This post-transcriptional regulation not only modulates expression of the tailspike protein—a key factor in host recognition and DNA injection—but also plays a pivotal role in superinfection exclusion, a mechanism that enables lysogenized bacteria to resist reinfection by the same or closely related phages. This exclusion contributes to the maintenance of the prophage state by preventing excessive phage replication and promoting the genomic stability of the host. Supporting this model, escape mutants of BTP1 were identified that could overcome the exclusion conferred by STnc6030. Sequencing of these variants revealed single nucleotide

polymorphisms clustered in the region antisense to the tailspike gene, highlighting the importance of specific base-pairing interactions in the regulatory mechanism [27]. Notably, while STnc6030 limits superinfection, it does not impair induction of the BTP1 prophage, suggesting a fine-tuned regulatory system that selectively restricts external infection without interfering with prophage activation [27].

Prophage-encoded ncRNAs also mediate non-virulence accessory functions, including the *sas* asRNA of phage P22 that induces a translational switch between distinct peptides encoded by the *sieB* gene and is critical to the function of the SieB superinfection-exclusion system [28]. The mechanism by which *sas* contributes to SIE involves its function as an asRNA that regulates the expression of the *sieB* gene, which encodes the SieB exclusion protein, that is known to inhibit lytic development of superinfecting phages such as P22 [28]. asRNA *sas* exerts its effect through base-pairing interactions with *sieB* mRNA, modulating its translation. Disruption of this RNA–RNA interaction, for instance by targeted mutations, leads to altered *sieB* expression and consequently affects superinfection efficiency. This suggests that precise base-pairing between *sas* and its target mRNA is a key element of the exclusion mechanism [28]. Additionally, the system displays a functional redundancy or overlap: even if *sieB* regulation is impaired, the Esc protein, which can be expressed by either the resident prophage or the incoming phage, can help bypass SieB-mediated exclusion. This indicates a complex regulatory balance between *sas*, SieB, and Esc.

5. Virulence and Stress Response

Bacteriophages play significant roles in modulating bacterial virulence and stress responses. When infecting bacteria, phages can trigger stress responses by introducing DNA damage or interfering with bacterial metabolic processes. In turn, bacterial cells activate defense mechanisms like the SOS response to cope with the phage infection. Additionally, some bacteriophages may influence bacterial virulence by carrying virulence factors or regulatory genes, which can enhance the pathogenicity of the host bacteria. This interaction between phages and bacteria shapes not only the bacterial stress response but also the potential for later disease transmission to humans [29].

For example, StxS functions as a regulatory sRNA that influences both Shiga toxin 1 production and the general stress response in *E. coli* O157:H7 [30]. It directly interacts with the *stx1AB* transcript, repressing Shiga toxin 1 production under lysogenic conditions. Additionally, StxS sRNA activates RpoS, the stationary-phase sigma factor, by binding to an activating seed sequence within the 5' UTR, a mechanism typically utilized by host-encoded sRNAs. This activation enhances bacterial growth at high cell densities under nutrient-limiting conditions, demonstrating how RNAs derived from phage regulation can obtain regulatory functions that impact host fitness [30].

The prophage-encoded small RNA SprY is another example of phage sRNAs, and it plays a crucial role in regulating virulence gene expression in *Staphylococcus aureus* [31]. SprY binds to RNAIII, a key virulence regulator, specifically targeting its 13th stem-loop, which is responsible for repressing multiple mRNA targets. By interacting with RNAIII, SprY increases the expression of certain virulence repressors while reducing hemolytic activity, ultimately decreasing *S. aureus* pathogenicity through an RNA sponge-like activity. mRNAs encoding the repressor of the Rot toxin and the extracellular complement-binding protein Ecb are among the targets whose expression is increased by SprY binding to RNAIII [31].

6. Host Metabolism and Environmental Adaptation

Bacteriophages rely on their host's cellular machinery by hijacking bacterial molecular systems for phage replication, transcription, and translation [32,33]. Phage-derived regu-

latory sRNAs play roles in controlling these processes, influencing bacterial metabolism while also modulating phage infection.

One such example is the 24B_1 microRNA-size molecule identified in *E. coli* after induction of Φ 24_B prophage [9,10]. 24B_1 alters the host's central carbon metabolism by binding to the *sdhB* mRNA and reducing intracellular ATP levels. Such a metabolic shift resembles the Warburg effect observed in cancer cells [11].

ipeX is another small RNA that plays a significant role in regulating gene expression in bacteria, particularly in the context of phage infection [34]. ipeX is known to regulate the expression of transcripts encoded by the core genome of the host bacteria and is synthesized after transcription from the phage's porin gene promoter, as part of the phage porin transcript. Specifically, ipeX has been shown to control the production of OmpC, a porin in *E. coli*, which is crucial for the bacterial outer membrane's permeability and function. The regulation by ipeX occurs through a mechanism that does not rely only on sequence complementarity with *ompC* mRNA. This suggests that additional factors may be involved in its regulatory function. The production of ipeX itself requires processing from a larger precursor transcript, a characteristic shared with eukaryotic microRNA [34,35].

Four sRNAs (*abiF*, *wcaG*, *manA*, *glnA*) were identified in the S-CREM1 cyanophage genome [36–38]. The *abiF* sRNA shares a conserved motif with *abiF* genes from various bacteria and bacteriophages, suggesting it may play a functional role during phage infection, though its exact regulatory role remains unconfirmed (Supplementary Table S1). Additionally, three *cis*-regulatory sRNAs, *wcaG*, *manA*, and *glnA*, with conserved motifs, were also found in other cyanophages. The *wcaG* sRNA is likely involved in regulating genes related to exopolysaccharide production, while the *manA* sRNA is associated with genes involved in nucleotide synthesis, carbohydrate metabolism, and photosynthesis. Lastly, the *glnA* sRNA is predicted to regulate genes related to nitrogen metabolism, including those encoding nitrogen regulatory proteins and transporters [36–38].

ϕ R1-37 is a bacteriophage that infects *Yersinia enterocolitica*, and its RNA plays a pivotal role during lytic infection, impacting both phage and host gene expression [39]. Ten novel RNA species were identified as being transcribed from the phage genome [39]. Nine of these (*misc_1-3* and *misc_5-10*) originate from the antisense strand, suggesting they function as asRNAs, but their exact role is unknown (Supplementary Table S1), while one, called *misc_4*, is encoded within an intragenic region and may interact with host mRNAs [39]. Bioinformatic analysis using TargetRNA2 predicted three potential host targets for *misc_4*: *ptr*, *tufA*, and *ddrA* that encode protease III, factor Tu, and the reactivation protein for propanediol utilization diol dehydratase, respectively [39].

Another study identified an asRNA linked to the *psbA* gene in the cyanophage S-PM2 [40]. This asRNA is designated as Cyanophage Functional RNA I (CfrI) and is encoded in the intergenic region between *psbA* and the gene coding for the homing endonuclease F-ChpI. This designation highlights its unique role in the context of cyanophage biology. One of the proposed functions of this asRNA is to prevent the formation of mobile group I introns within the *psbA* gene of cyanophages. This could be crucial for maintaining the integrity of the *psbA* gene during the phage's lifecycle. The presence of a gene encoding this asRNA at the 3' end of the *psbA* gene in various cyanophages implies that such a regulatory mechanism may be evolutionarily conserved [40].

Bacterial motility may contribute to virulence or improve bacterial survival in various environments. Esr41 is a small RNA from an intergenic region unique to the enterohemorrhagic *E. coli* (EHEC) O157:H7 Sakai strain, which is absent in the genome of the nonpathogenic *E. coli* K-12 [41]. The sRNA enhances the expression of the *fliC* gene, leading to increased bacterial cell motility [41]. Motility assays showed that overexpression of Esr41 from a multicopy plasmid significantly increased the motility of EHEC. This is linked to

the upregulation of *fliC*, which encodes the major flagellar filament protein, FliC. As a *trans*-acting, Hfq-binding sRNA, Esr41 was proposed to regulate motility-related genes through base-pairing interactions with target mRNAs, particularly within the 5' untranslated region [41]. Its role in motility enhancement is conserved across different *E. coli* strains, including the non-pathogenic *E. coli* K-12, suggesting a broader regulatory function. Additionally, Esr41 likely participates in a complex motility regulation network, interacting with other sRNAs and transcription factors to coordinate flagellar gene expression in response to environmental changes, highlighting its potential role in bacterial adaptation and pathogenicity [41].

7. Regulation of Gene Expression

Bacteriophages, as viruses, may manipulate key pathways in their bacterial hosts at the transcriptional level, optimizing conditions for their replication. By influencing gene expression, phages create optimal conditions that favor their propagation [42].

IsrK sRNA, encoded by the Gifsy-1 prophage, functions as both a small RNA and a messenger RNA, modulating key bacterial processes [43]. It regulates gene expression by activating *antQ*, a gene encoding an antiterminator protein AntQ, located upstream of *isrK*. Raised levels of IsrK sRNA have been shown to inhibit *Salmonella* growth, an effect linked to the expression of *antQ*. The protein disrupts transcription termination, creating conflicts between transcription and replication that lead to DNA damage. When AntQ accumulates at high levels, bacterial growth is suppressed, ultimately resulting in cell death [43]. Then, stress occurs within the bacterial cell and ultimately results in a growth decrease and leads to cell death. Additionally, AntQ promotes the formation of R-loops, hybrid RNA–DNA structures that can initiate DNA replication independently of the origin of replication (*oriC*). This uncontrolled replication leads to DNA damage, further contributing to its cytotoxic effects. Bacterial cells expressing *antQ* also exhibit condensed chromatin morphology, suggesting a broader impact on genome organization. Despite these harmful effects, bacterial cells have mechanisms to counteract AntQ toxicity [43]. The co-expression of transcription termination factors such as Rho and RNase H helps restore normal transcription processes, resolving R-loops, and maintaining genomic integrity, thereby mitigating AntQ-induced stress. Experimental data showed that *antQ* expression significantly reduced bacterial survival rates [43]. Together, these findings highlight AntQ as a potent regulator of bacterial physiology, capable of disrupting transcription, damaging DNA, and severely impacting cell survival, whose action is controlled by sRNA [43].

Moreover, the IsrK sRNA plays a crucial role in modulating gene expression, particularly in the regulation of open reading frame *orf45* and *anrP* gene. IsrK is essential for the activation of the *anrP* gene, which is located downstream of *orf45*. This activation occurs through a mechanism involving the translation of *orf45*, suggesting that IsrK enhances downstream gene expression by facilitating *orf45* translation. In other words, the regulation of *anrP* gene by IsrK occurs by translational coupling, in which the translation of *orf45* is a necessary point for *anrP* expression. In effect, IsrK promotes the translation of *orf45*, thereby enabling the later translation of gene *anrP*. Comparative sequence analysis indicates that while the nucleotide sequence of *orf45* is conserved among the *Enterobacteriaceae* family, its amino acid sequence exhibits variability. This suggests that the regulatory interaction between *orf45* and IsrK may be an important feature conserved across different bacterial species [43].

Roles of small regulatory RNAs that are derived from bacteriophages were discussed recently [44,45], particularly focusing on their roles in the regulation of gene expression in EHEC. AgvB sRNA is encoded by the Sp5 prophage of enterohemorrhagic *E. coli*. Unlike typical sRNAs, it does not function as an antisense regulator but instead inhibits the activity

of another sRNA, GcvB, encoded in the *E. coli* chromosome. By mimicking the mRNA targets of GcvB, AgvB counteracts its regulatory effects, allowing the expression of genes otherwise repressed by GcvB. This global regulator controls the translation of up to 1% of transcripts [44]. AgvB relieves the translational repression of the dipeptide transporter DppA, which GcvB normally represses. This indicates that AgvB plays a crucial role in regulating amino acid transport and metabolism in enterohaemorrhagic *E. coli* [44,45].

Another identified sRNA is AsxR from the Shiga-toxin-2-encoding phage of *E. coli* O157:H7 Sakai strain. This molecule functions as an asRNA that increases the translation of ChuS, a heme oxygenase, by destabilizing the small regulatory FnrS RNA [44]. FnrS typically represses ChuS translation by binding to the Shine–Dalgarno sequence, but AsxR disrupts FnrS stability by binding to its Rho-independent terminator stem-loop. This interaction reduces FnrS levels and its association with the Hfq chaperone, thereby soothing the repression of ChuS translation [44].

The c4 RNA is a regulatory asRNA found in bacteriophages P1 and P7, playing a critical role in controlling gene expression by targeting *ant* mRNA, which encodes anti-repressor protein [46]. Its primary function is to inhibit the synthesis of anti-repressors. This mechanism is essential for the heteroimmunity observed between P1 and P7, allowing them to prevent co-infection of the same bacterial host. c4 RNA operates through sequence-specific binding to complementary regions of the *ant* mRNA, particularly around the ribosome binding site, thereby blocking translation [46]. The function of c4 RNA is strongly dependent on its secondary structure, with the complementary sequences likely positioned within RNA loops to facilitate efficient binding. Research into c4 RNA has also highlighted its potential as a prototype of a new class of regulatory RNAs, suggesting that additional, similarly functioning asRNAs might exist. Overall, c4 RNA is a key regulatory element that modulates phage immunity and gene expression through sophisticated antisense interactions [46].

8. Concluding Remarks

Bacteriophage-derived small RNAs represent a newly emerging layer of genetic regulation in bacteria, influencing essential cellular functions and the complex interactions between bacteriophages and their hosts (Figure 2). These sRNAs contribute to the regulation of bacterial metabolism, stress response, and virulence by modulating gene expression, often through mechanisms that resemble eukaryotic virus sRNAs [35]. Their regulatory functions can significantly impact bacterial physiology, shaping the outcomes of phage infection and integration. A functional classification of phage-derived small RNAs, based on cellular processes they modulate, is presented in Table 1.

One key role of phage-derived sRNAs is their ability to modify host metabolism, which resembles microRNA in herpesviruses in eukaryotic cells [11]. For instance, the 24B_1 sRNA from bacteriophage Φ 24_B influences central carbon metabolism by interacting with *sdhB* mRNA, ultimately reducing ATP levels within the bacterial cell [10]. Beyond metabolic regulation, these sRNAs are also implicated in stress responses. While their precise functions are not always fully explained, the well-established role of *trans*-encoded sRNAs in bacterial stress adaptation suggests that some phage-derived sRNAs may similarly contribute to host resilience under adverse conditions [4].

Table 1. Functional classification of phage-derived small RNAs based on cellular processes.

Cellular Process	sRNA Name	Function	Host	Phage	Size (nt)	sRNA Acting Type	Reference
Phage Replication and Lysogeny Control	VpdS	Regulates phage replication in <i>V. cholerae</i> , linking quorum sensing to phage life cycle.	<i>Vibrio cholerae</i>	VP882	~50	trans	[16]
	PreS (LPR1)	Regulates the expression of <i>dnaN</i> gene, optimizing conditions for λ phage propagation.	<i>Escherichia coli</i>	λ	~88	trans	[17]
	24B_1	Promotes lysogeny by enhancing the expression of <i>cl</i> , <i>cII</i> , and <i>cIII</i> genes and altering host metabolism.	<i>Escherichia coli</i>	Φ 24 _B	20	trans	[9,10]
	PAK_P4as15	Acts as a translational repressor of the endolysin gene, delaying host cell lysis.	<i>Pseudomonas aeruginosa</i>	PAK_P4	~550	cis	[18]
	CfrI	Prevents mobile intron formation in <i>psbA</i> gene of cyanophages, ensuring stability.	<i>Synechococcus</i> sp. WH7803	S-PM2	~225	cis	[38]
	sar	Pairs with <i>ant</i> mRNA and inhibits antirepressor synthesis.	<i>Salmonella enterica</i> serovar Typhimurium	P22	68/69	trans	[19]
	OOP	Controls <i>cII</i> gene expression and influences the lysis–lysogeny.	<i>Escherichia coli</i>	λ and Stx phages	77	cis	[15]
	aQ	Regulates <i>Q</i> gene expression	<i>Escherichia coli</i>	λ	220	cis	[20]
Bacterial Cell Division and Morphology	DicF	Inhibits expression of <i>ftsZ</i> gene and cell division—promotes cell filamentation under anaerobic conditions.	<i>Escherichia coli</i>	Qin	53	trans	[21–25]
Superinfection Exclusion	STnc6030	Mediates superinfection exclusion of phage BTP1.	<i>Salmonella enterica</i> serovar Typhimurium D23580	BTP1	786	cis	[27]
	sas	Induces a translational switch in <i>sieB</i> , crucial for the SieB superinfection-exclusion system.	<i>Salmonella enterica</i> serovar Typhimurium	P22	105	cis	[28]
Virulence and Stress Response	SprY	Regulates RNAPIII activity, reducing the virulence and hemolytic activity of the bacterial cell.	<i>Staphylococcus aureus</i>	prophage ϕ 12	~125	trans	[31]
	StxS	Represses Shiga toxin 1 production and activates <i>rpoS</i> , enhancing stress resistance in <i>E. coli</i> O157:H7.	<i>Escherichia coli</i> O157:H7	Stx Φ	74	trans	[30]
	Esr41	Stimulates <i>fliC</i> expression, increasing bacterial motility, which may influence virulence.	<i>Escherichia coli</i> O157:H7 str. Sakai	Sakai prophage-like element 1 (SpLE1)	~70	trans	[39]

Table 1. Cont.

Cellular Process	sRNA Name	Function	Host	Phage	Size (nt)	sRNA Acting Type	Reference
Host Metabolism and Environmental Adaptation	24B_1	Alters carbon metabolism by binding to <i>sdhB</i> mRNA, reducing ATP levels.	<i>Escherichia coli</i>	Φ24 _B	20	<i>trans</i>	[9,10]
	ipeX	Regulates the expression of <i>ompC</i> gene, affecting membrane permeability.	<i>Escherichia coli</i>	cryptic prophage DLP12	167	<i>cis</i>	[34]
	wcaG	Probably regulates the expression of genes linked to exopolysaccharide production.	<i>Synechococcus</i>	S-CREM1	97	<i>cis</i>	[36–38]
	manA	Associated with nucleotide synthesis, carbohydrate metabolism, and photosynthesis.	<i>Synechococcus</i>	S-CREM1	214	<i>cis</i>	[36]
	glnA	Probably regulates the expression of genes related to nitrogen metabolism.	<i>Synechococcus</i>	S-CREM1	98	<i>cis</i>	[36]
	misc_4	Predicted to interact with host mRNAs, including <i>ptr</i> , <i>tufA</i> , and <i>ddrA</i> .	<i>Yersinia enterocolitica</i>	φR1-37	296	<i>trans</i>	[37]
Regulation of Gene Expression	AgvB (EcOnc01)	Counteracts the regulatory effects of the bacterial GcvB sRNA by mimicking its mRNA targets.	<i>Escherichia coli</i> O157:H7 str. Sakai	lambdoid prophage Sp5	~60	<i>trans</i>	[44,45]
	IsrK	Regulates transcription termination through <i>antQ</i> , affecting <i>Salmonella</i> growth. Has a dual role as sRNA and mRNA.	<i>Salmonella typhimurium</i>	prophage Gifsy-1	217	<i>cis</i> and <i>trans</i>	[43]
	c4	Controls gene expression by targeting <i>ant</i> mRNA, which encodes antirepressor proteins.	<i>Escherichia coli</i>	P1 and P7	77	<i>trans</i>	[46]
	EcOnc02 (AsxR)	Derepresses a heme oxygenase. Enhances ChuS translation by destabilizing the FnrS sRNA.	<i>Escherichia coli</i> O157:H7 str. Sakai	lambdoid prophage Sp5	~54	<i>trans</i>	[44]

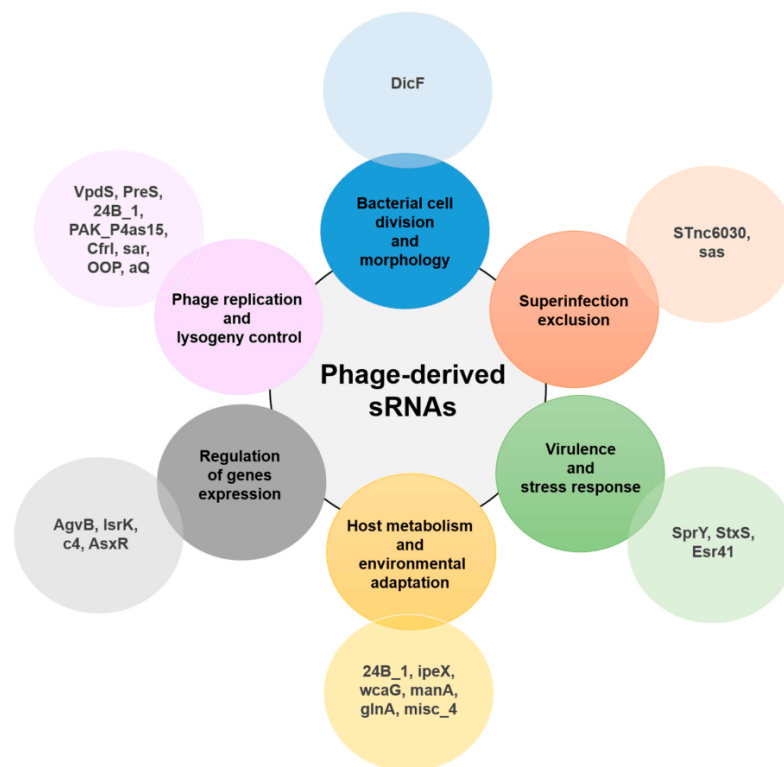


Figure 2. Roles of phage-encoded sRNAs in different cellular processes: The experimentally validated sRNAs are depicted along with the respective biological processes.

Virulence regulation is another crucial aspect of phage sRNAs' activity. In *S. aureus*, the prophage-encoded SprY sRNA modulates the expression of virulence factors by binding to RNAIII, a central regulatory RNA involved in pathogenesis [31]. Similarly, the phage-derived Esr41 sRNA enhances bacterial motility in enterohemorrhagic *E. coli* [41]. These examples highlight how phage sRNAs can actively shape the interaction between bacteria and their environment, influencing host adaptability and infectivity.

The molecular mechanisms employed by phage-derived sRNAs are diverse, often mirroring those of bacterial regulatory RNAs. Many such regulatory molecules function as asRNAs, pairing with complementary mRNA sequences to regulate translation and stability. A notable example is PAK_P4as15, an asRNA from phage PAK_P4 that may repress endolysin translation, potentially delaying bacterial cell lysis [18]. Additionally, some sRNAs interact with RNA-binding proteins, such as the chaperone Hfq, which stabilizes sRNAs and eases their regulatory interactions. For instance, the phage-derived VpdS [16] and PreS [17] sRNAs require Hfq for efficient mRNA binding, while AgvB competes with bacterial sRNAs for Hfq binding, interfering with host regulatory pathways [44].

A particularly interesting aspect of phage sRNAs is their resemblance to eukaryotic microRNAs, both in size and maturation (from original transcripts) and function, like 24B_1 sRNA. Not only is its action similar to microRNAs of herpesviruses but also 24B_1 is processed from a longer 80 nt precursor that resembles the biogenesis of eukaryotic microRNAs [9–11]. Although bacteria lack the complex RNA maturation system found in eukaryotes [35], the Hfq protein was demonstrated to be involved in the maturation process [10].

Beyond their roles in modulating host physiology, phage-derived sRNAs significantly impact the bacteriophage life cycle by regulating the lysogenic–lytic switch. These

molecules help to determine whether a phage remains integrated within the host genome or initiates induction and replication. For example, 24B_1 sRNA promotes lysogeny in phage Φ 24_B [9,10]. VpdS, encoded by the temperate phage VP882, plays a key role in triggering phage replication in *V. cholerae* in response to quorum-sensing signals [16]. Additionally, certain phage sRNAs optimize conditions for their replication by targeting bacterial genes critical for DNA synthesis, such as the *dnaN* gene targeted by PreS in λ phage [17]. Other sRNAs, like PAK_P4as15, may extend the time frame for viral replication by delaying host cell lysis [18].

The similarities between phage-derived sRNAs and eukaryotic microRNAs extend beyond function to evolutionary implications. The presence of mobile genetic elements, such as bacteriophages, could have facilitated the spread and improvement of these regulatory mechanisms. Additionally, while the Hfq protein in bacteria is not homologous to eukaryotic Argonaute proteins, it serves a similar function in presenting sRNAs to their targets, further empowering the functional parallels between these systems [35].

Bacteriophage-derived sRNAs represent an important but still largely unexplored field of bacterial and phage regulation, influencing host metabolism, stress adaptation, virulence, and the phage cycles. Their functional similarities to eukaryotic microRNAs highlight the unconventional view that microRNA-based gene regulation is not exclusive to eukaryotes. Thus, studying phage sRNAs could provide key insights into microbial genetics and lead to new applications in biotechnology, medicine, and treatments such as phage therapy.

The findings discussed in this review article illustrate the complex interactions between phage-derived sRNAs and their host bacteria, emphasizing their potential impact on bacterial behavior and pathogenicity. The structural and functional parallels between phage sRNAs and eukaryotic microRNAs highlight how phage-derived sRNAs can shape bacterial transcriptional networks in a way that eukaryotic viruses do in their hosts.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/v17060834/s1>, Supplementary Table S1. Phage-derived sRNA molecules that have been identified; however, their specific functional roles within the cell remain undefined.

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Suplement do pracy nr 1

Supplementary Table S1. Phage-derived molecules that have been identified; however, their specific functional roles within the cell remain undefined.

sRNA name	Host	Phage	Size (nt)	Potential function	References
LPR2	<i>Escherichia coli</i>	λ	~63	Associated with the lytic cycle	[17]
6S	<i>Escherichia coli</i>	λ	~194 and ~121	Associated with the lytic cycle	[17]
PAK_P4as06 RNA	<i>Pseudomonas aeruginosa</i>	PAK_P4	200	Unknown	[18]
PAK_P4as07 RNA	<i>Pseudomonas aeruginosa</i>	PAK_P4	200	Unknown	[18]
PAK_P4as14 RNA	<i>Pseudomonas aeruginosa</i>	PAK_P4	200	Unknown	[18]
PAK_P4as16 RNA	<i>Pseudomonas aeruginosa</i>	PAK_P4	200	Unknown	[18]
STnc6000	<i>Salmonella enterica</i> serovar Typhimurium D23580	BTP1	-	Unknown	[27]
STnc6080	<i>Salmonella enterica</i> serovar Typhimurium D23580	BTP1	-	Unknown	[27]
STnc6010	<i>Salmonella enterica</i> serovar Typhimurium D23580	BTP1	-	Unknown	[27]
STnc6020	<i>Salmonella enterica</i> serovar Typhimurium D23580	BTP1	-	Unknown	[27]
STnc6030	<i>Salmonella enterica</i> serovar Typhimurium D23580	BTP1	-	Unknown	[27]
STnc6040	<i>Salmonella enterica</i> serovar Typhimurium D23580	BTP1	-	Unknown	[27]
STnc6050	<i>Salmonella enterica</i> serovar Typhimurium D23580	BTP1	-	Unknown	[27]
STnc6060	<i>Salmonella enterica</i> serovar Typhimurium D23580	BTP1	-	Unknown	[27]

STnc6070	<i>Salmonella enterica</i> serovar Typhimurium D23580	BTP1	-	Unknown	[27]
misc_1	<i>Yersinia enterocolitica</i>	φR1-37	129	Unknown	[40]
misc_2	<i>Yersinia enterocolitica</i>	φR1-37	394	Unknown	[40]
misc_3	<i>Yersinia enterocolitica</i>	φR1-37	320	Unknown	[40]
misc_5	<i>Yersinia enterocolitica</i>	φR1-37	102	Unknown	[40]
misc_6	<i>Yersinia enterocolitica</i>	φR1-37	340	Unknown	[40]
misc_7	<i>Yersinia enterocolitica</i>	φR1-37	350	Unknown	[40]
misc_8	<i>Yersinia enterocolitica</i>	φR1-37	310	Unknown	[40]
misc_9	<i>Yersinia enterocolitica</i>	φR1-37	174	Unknown	[40]
misc_10	<i>Yersinia enterocolitica</i>	φR1-37	324	Unknown	[40]
EcOnc03	<i>Escherichia coli</i> O157:H7 str. Sakai	Lambdoid prophage encoded	~54	Unknown	[45]
EcOnc04	<i>Escherichia coli</i> O157:H7 str. Sakai	Lambdoid prophage encoded	~222	Unknown	[45]
EcOnc05	<i>Escherichia coli</i> O157:H7 str. Sakai	Lambdoid prophage encoded	~222	Unknown	[45]
EcOnc06	<i>Escherichia coli</i> O157:H7 str. Sakai	Lambdoid prophage encoded	~177	Unknown	[45]
EcOnc08/21/42	<i>Escherichia coli</i> O157:H7 str. Sakai	Lambdoid prophage encoded	~271	Unknown	[45]
EcOnc13	<i>Escherichia coli</i> O157:H7 str. Sakai	Lambdoid prophage encoded	~187	Unknown	[45]
EcOnc18	<i>Escherichia coli</i> O157:H7 str. Sakai	Lambdoid prophage encoded	~37	Unknown	[45]
EcOnc22	<i>Escherichia coli</i> O157:H7 str. Sakai	Lambdoid prophage encoded	~85	Unknown	[45]
EcOnc27	<i>Escherichia coli</i> O157:H7 str. Sakai	Lambdoid prophage encoded	~247/235	Unknown	[45]
EcOnc32	<i>Escherichia coli</i> O157:H7 str. Sakai	Lambdoid prophage encoded	~112	Unknown	[45]
EcOnc33	<i>Escherichia coli</i> O157:H7 str. Sakai	Lambdoid prophage encoded	~71	Unknown	[45]
EcOnc50	<i>Escherichia coli</i> O157:H7 str. Sakai	Lambdoid prophage encoded	~78	Unknown	[45]
EcOnc38	<i>Escherichia coli</i> O157:H7 str. Sakai	Lambdoid prophage encoded	~256	Unknown	[45]

Oświadczenia współautorów

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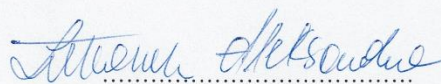
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kierowaniu jednym z projektów badawczych, w ramach których powstała ta praca, dyskusjach nad układem i formatem manuskryptu, a także wprowadzeniu poprawek do wstępnej i ostatecznej wersji manuskryptu.



prof. dr hab. Grzegorz Węgrzyn

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B. Nejman-Faleńczyk
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PODPIS

Praca nr 2

Bacteriophage-encoded 24B_1 molecule resembles herpesviral microRNAs and plays a crucial role in the development of both the virus and its host

Bloch, S[#]., Lewandowska, N[#]., Zwolenkiewicz, J., Mach, P., Łukasiak, A., Olejniczak, M., Donaldson, L. W., Węgrzyn, G., & Nejman-Faleńczyk, B.

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RESEARCH ARTICLE

Bacteriophage-encoded 24B_1 molecule resembles herpesviral microRNAs and plays a crucial role in the development of both the virus and its host

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Abstract

The 24B_1 small non-coding RNA molecule has been identified in *Escherichia coli* after induction of Shiga toxin-converting bacteriophage Φ24_B. In this work, we focused on its direct role during phage and bacterial host development. We observed that in many aspects, this phage sRNA resembles herpesviral microRNAs. Similar to microRNAs, the mature 24B_1 is a short molecule, consisting of just 20 nucleotides. It is generated by cleaving the 80-nt long precursor transcript, and likely it undergoes a multi-step maturation process in which the Hfq protein plays an important role, as confirmed by demonstration of its binding to the 24B_1 precursor, but not to the 24B_1 mature form. Moreover, 24B_1 plays a significant role in maintaining the prophage state and reprogramming the host's energy metabolism. We proved that overproduction of this molecule causes the opposite physiological effects to the mutant devoid of the 24B_1 gene, and thus, favors the lysogenic pathway. Furthermore, the 24B_1 overrepresentation significantly increases the efficiency of expression of phage genes coding for proteins CI, CII, and CIII which are engaged in the maintenance of the prophage. It seems that through binding to mRNA of the *sdhB* gene, coding for the succinate dehydrogenase subunit, the 24B_1 alters the central carbon metabolism and causes a drop in the ATP intracellular level. Interestingly, a similar effect, called the Warburg switch, is caused by herpesviral microRNAs and it is observed in cancer cells. The advantage of the Warburg effect is still unclear, however, it was proposed that the metabolism of cancer cells, and all rapidly dividing cells, is adopted to convert nutrients such as glucose and glutamine faster and more efficiently into biomass. The availability of essential building blocks, such as nucleotides, amino acids, and lipids, is crucial for effective cell proliferation which in turn is essential for the prophage and its host to stay in the lysogenic state.

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Introduction

Shiga toxin-producing *Escherichia coli* (STEC) bacteria are highly harmful to humans as they contain lambdoid prophages (called Stx phages) bearing the *stx* genes coding for Shiga toxins, the major agents responsible for the development of severe diseases. Stx phages bear many similarities in the life cycle and genomic organization to bacteriophage λ , the most-investigated member of this family [1, 2]. The Stx phage lifecycle begins when the phage adsorbs to the surface of the host bacteria and injects its linear double-stranded DNA into the cell. At this stage, depending on the intracellular conditions, phage may develop according to one of two alternative pathways, lysogenic or lytic [3, 4]. When the lysogenic pathway is chosen, phage DNA is incorporated into *E. coli* genome (forming a prophage), replicated together with the bacterial genome, and then transmitted to daughter cells during cell division. The excision of the phage genome occurs following prophage induction. This may be caused by different factors like UV, antibiotics, or hydrogen peroxide that is naturally produced in the human body [5–9]. As a consequence, viral DNA starts to replicate separately from the host bacterial chromosome as an extrachromosomal element [10, 11]. This leads to the synthesis of phage structural and lysis proteins. In effect, the assembly of multiple new phage particles, disruption of the bacterial host cell, and release of large amounts of Shiga toxins and Stx progeny phages occur [12, 13].

The progressive production of Shiga toxins causes bloody diarrhea as the first symptom of human infection. Other symptoms like abdominal pain and vomiting may also occur. Symptoms of food poisoning maintain around 10 days, however, 15–20% of patients infected with these pathogens progress to severe complications such as hemolytic uremic syndrome, thrombocytopenia, or hemorrhagic colitis. Children under five years old and the elderly are most susceptible to severe complications with mortality as high as 87% [14–16]. The main problem of human infection by STEC is a lack of known effective treatment strategies. There is serious doubt of using antibiotics, as they are prophage inducers that indirectly stimulate the expression of genes coding for Shiga toxins and consequently, enhance the severity of disease symptoms [6, 16–18].

The severity of the infection and the development of complications depend on the amount of Shiga toxin produced by bacteria. Importantly, the expression of *stx* genes is tightly linked to lytic development which starts after prophage induction. Otherwise, during lysogeny, the majority of phage genes (including these coding for Shiga toxins) are suppressed. In general, the switch between lysogenic and lytic pathway is based on the activation and inactivation of the major phage repressor of the lytic development (called CI in the case of lambdoid phages). This DNA-binding repressor is involved in the inhibition of the transcription from the main phage promoters p_L and p_R . The binding of the repressor to the operator sites is a well-known mechanism of negative regulation that inhibits transcriptional initiation and thus suppresses the expression of the genes essential for the lytic cycle, synthesis of toxins, and phage lytic development at all. Before a phage enters the lytic pathway, repressors must be inactivated [19]. The best known mechanism responsible for the inactivation of the phage repressors in lambdoid phages involves the host SOS system that can be induced by physiological changes in the host cells, UV light irradiation, hydrogen peroxide treatment, or antibiotics. In most cases, the inducers of the SOS response cause DNA damage which results in the activation of the RecA protein and subsequent degradation of both the bacterial LexA protein and the phage-encoded CI repressor. The second mechanism to control the repressor activity uses phage-encoded antirepressor protein (frequently called Ant) which acts through the competitive binding with the repressor to the same operator-binding site. Some of the identified antirepressor genes are under the direct control of the LexA, however, some reports indicate that

also small non-coding RNAs may be involved in this regulation. For example, the Ant antirepressor synthesis is regulated during the development of bacteriophage P22 by the small anti-sense RNA named Sar. Notably, this sRNA molecule acts in *trans* by binding to the ribosome binding site of the Ant mRNA and inhibits Ant production at a post-transcriptional level [20, 21]. Similarly, in phage N15 which is a temperate virus of *E. coli* related to lambdoid phages, the antirepressor gene is negatively regulated by a small RNA, named CA [22].

Recently, our group discovered 24B_1, a 20-nt microRNA-like molecule, after induction of Shiga toxin-converting bacteriophage Φ24_B [23]. Importantly this molecule has been shown to stimulate the lysogenic pathway of phage lifecycle in *E. coli* bacteria. We speculated that 24B_1 may act as a regulator of D_ant antirepressor gene expression and thus indirectly control the activity of the repressor of the phage lytic cycle. Previously, we have tested aspects of the development of bacteriophage Φ24_B bearing a deletion of the sequence encoding the 24B_1 precursor molecule, that is located between genes *lom* and *vb_24B_43* of the Φ24_B phage genome. The obtained results indicated that the lytic developmental pathway was more efficient in the mutant phage lacking the 24B_1 molecule [23]. These promising results prompted us to take a closer look at the 24B_1 role during the phage development in bacteria. In this report, we decided to analyze the influence of the overproduced 24B_1 molecule on the development of the phage as well as its host. Manipulation of the phage lysogenic-lytic decision through microRNA-like particles offers a new way to understand developmental pathways and identify possible therapeutic interventions against pathogenic *E. coli* infections.

Materials and methods

Bacterial strains, bacteriophages, and plasmids

E. coli MG1655 strain and its derivatives employed in this work, are presented in Table 1. The deletion mutant, lacking the sequence of the small, microRNA-size, 24B_1 molecule was constructed as described previously by [23] by using *E. coli* MG1655 (Φ24_B) strain and the Quick and Easy *E. coli* Gene Deletion Kit (Gene Bridges, Heidelberg, Germany). Phages Φ24_B [24] and Φ24_BΔ24B_1 [23] were from the collection of the Department of Molecular Biology of the University of Gdansk (Poland). Plasmid pUC18 (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used as a control variant in all experiments presented in this work. Plasmid pUC18_24B_1, bearing the 189-nt region of Φ24_B genome encompassing the identified 24B_1 sequence, was described by us earlier [23]. The 24B_1 transcript production from pUC18_24B_1 plasmid was also confirmed previously [23]. The plasmid bearing an additional copy of the 24B_1 gene was incorporated into the *E. coli* MG1655 strain and used to overproduce the 24B_1 molecule in cells infected with wild-type phage Φ24_B.

Media and growth conditions

For all experiments, *E. coli* bacteria were routinely cultured in the Luria-Bertani (LB) medium (EPRO, Władysławowo, Poland) at 37°C with aeration achieved by shaking. The LB broth, supplemented with 0.7% or 1.5% bacteriological agar (BTL, Łódź, Poland), was used as a top

Table 1. *E. coli* MG1655 strain and its derivatives.

<i>E. coli</i> strains	Relevant genotype or description	References
MG1655	F [−] λ [−] <i>ilvG rfb-50 rph-1</i>	[25]
MG1655 (Φ24 _B)	MG1655 bearing Φ24 _B prophage	[26]
MG1655 (Φ24 _B Δ24B_1)	MG1655 bearing Φ24 _B prophage with deletion of the region encompassing the 80-nt long sequence of the predicted secondary structure of 24B_1	[23]

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or bottom agar, respectively. The Petri dishes with the solid medium were incubated at 37°C for 20 h.

Preparation of phage lysate

To obtain purified $\Phi 24_B$ or $\Phi 24_B\Delta 24B_1$ particles, bacteria lysogenic for tested bacteriophages were grown in LB medium to an OD_{600} of 0.1. Then, mitomycin C (EPRO, Władysławowo, Poland) was added to the flask to a final concentration of 1 $\mu\text{g/ml}$ to provoke prophage induction. The mixture was incubated at 37°C on a rotary shaker at the rate of 200 RPM for about 18 h. Following the lysis of host cells, the lysate was treated with 4% chloroform for 15 minutes and cleared of bacterial debris by centrifugation (2000 x g, 10 min, 4°C). The supernatant containing phage virions was concentrated in the presence of 10% polyethylene glycol 8000 (PEG8000; EPRO, Władysławowo, Poland) on a magnetic stirrer at 4°C for about 20 h. The homogenous mixture was centrifuged (8000 x g, 20 min, 4°C) and the white phage pellet was resuspended in TM buffer (10 mM Tris-HCl, 10 mM MgSO_4 , pH 7.2). The suspension was extracted three times with chloroform (Chempur, Piekary Śląskie, Poland) after centrifugation (2000 x g, 10 min, 4°C). Finally, the upper aqueous layer with the virus particles was collected and the phage titer was determined according to the procedure described in the next section. The phage stock was stored at 4°C.

Determination of the concentration of phage particles in the solution

Phage lysate titration was performed on the standard Petri dishes (Alchem, Toruń, Poland) according to the double overlay plaque assay described by [27]. The top layer was prepared by mixing 2 ml of a prewarmed soft LB agar (0.7%) with 1 ml of the indicator *E. coli* MG1655 strain culture. Following supplementation of the mixture with MgSO_4 (Chempur, Piekary Śląskie, Poland) and CaCl_2 (Chempur, Piekary Śląskie, Poland) to the final concentration of 10 mM, it was poured onto solid bottom LB agar (1.5%) with a sublethal concentration of chloramphenicol (2.5 $\mu\text{g/ml}$; EPRO, Władysławowo, Poland). Supplementation of the bottom LB agar with appropriate antibiotic was effective in increasing the size of plaques formed by Stx phages. To determine the number of virus particles per ml suspension (PFU/ml), serial 10-fold dilution was prepared in TM buffer (10 mM Tris-HCl, 10 mM MgSO_4 , pH 7.2). Then, 2.5 μl of each dilution was spotted onto the surface of the top LB agar. Petri dishes were incubated at 37°C for 20 h and the phage concentration was quantified on the basis of the number of plaques.

Determination of lysis time of host bacteria

Host bacteria were cultivated in LB medium with 10 mM MgSO_4 (Chempur, Piekary Śląskie, Poland) and 10 mM CaCl_2 (Chempur, Piekary Śląskie, Poland) at 37°C to an OD_{600} of 0.2. The phage stock solution was added to the flask with bacterial culture to an m.o.i. of 2. The lysis kinetics of infected bacteria were monitored spectrophotometrically by measuring OD_{600} over a period of 300 min. Additionally, the number of bacterial cells per ml (CFU/ml) was determined. To calculate the number of viable bacterial cells after phage infection, 100 μl samples were harvested at appropriate times, and their serial 10-fold dilutions were prepared in 0.85% NaCl (Chempur, Piekary Śląskie, Poland). Then, 40 μl of each dilution was spread onto the bottom LB agar supplemented with ampicillin to a final concentration of 50 $\mu\text{g/ml}$ (EPRO, Władysławowo, Poland). After overnight incubation at 37°C, the bacterial colonies were counted, and the CFU/ml was determined.

Survival of host bacteria after phage infection in pro-lytic or pro-lysogenic conditions

The procedure described previously was used, with slight modifications [23]. Briefly, host cells were cultured with aeration at 37°C to an OD₆₀₀ of 0.2. Then, 1 ml of the sample was centrifuged (2000 × g, 10 min, 4°C) and the pellet was suspended in (i) 1 ml of LB medium enriched with 10 mM MgSO₄ and CaCl₂ (pro-lytic conditions) or (ii) 1 ml of TCM buffer (pro-lysogenic conditions; 10 mM Tris-HCl, 10 mM MgSO₄, 10 mM CaCl₂; pH 7.2). Tested bacteriophages were added to the bacterial suspension to an m.o.i. of 5. In the control variant of the experiment, TM buffer (10 mM Tris-HCl, 10 mM MgSO₄; pH 7.2) was used instead of phage lysate. The mixture was incubated for 15 min at 37°C, and then serial 10-fold dilutions were prepared in 0.85% NaCl (Chempur, Piekary Śląskie, Poland). Then, 40 µl of each dilution was spread onto the bottom LB agar supplemented with ampicillin to a final concentration of 50 µg/ml. Following overnight incubation at 37°C, the fraction of surviving bacteria was calculated relative to the control variant not infected with tested phages.

Estimation of efficiency of lysogenization

Lysogens among survivors were identified according to the procedure described by [28], with one minor modification. Briefly, bacterial colonies obtained after phage infection were passaged separately, each in a well of a 96-well plate filled with 200 µl of LB medium. The suspensions were incubated with shaking at 37°C to an OD₆₀₀ of 0.1. For estimation of the number of lysogens among tested bacterial cells, mitomycin C was added to each well to a final concentration of 1 µg/ml (this antibiotic had been demonstrated previously to induce prophages in *E. coli* bacteria [23, 26, 29]). The plates were then incubated for an additional 3 h. Afterward, 12 µL of chloroform was added to each well and the plates were centrifuged (2000 × g, 10 min, 4°C). Then, 2.5 µl of water phase was spotted onto double-layer LB agar supplemented with 2.5 µg/ml of chloramphenicol. The plates were incubated at 37°C for 20 h. A colony was determined as lysogenic if plaques were formed on a bacterial lawn. The efficiency of lysogenization was calculated as a percent of lysogens among all tested bacterial colonies that survived phage infection.

Intracellular lytic development of tested phages in host bacteria

One-step growth experiments were performed as described previously by [23]. Host bacteria were cultured in LB medium supplemented with 10 mM MgSO₄ (Chempur, Piekary Śląskie, Poland) and 10 mM CaCl₂ (Chempur, Piekary Śląskie, Poland) at 37°C to an OD₆₀₀ of 0.2. Then, 10 ml of bacterial suspension was centrifuged (3000 × g, 10 min, 4°C) and the obtained pellet was suspended in 1 ml of fresh LB medium with 3 mM NaN₃ (Merck, Darmstadt, Germany). The phage lysate was added to the host culture to a multiplicity of infection (m.o.i.) of 0.05. Following 10-min incubation at 37°C, the mixture was diluted 10-fold in LB medium supplemented with 3 mM NaN₃ and centrifuged (3000 × g, 10 min, 4°C). The supernatant containing free, unadsorbed virions was discarded and the bacterial pellet was suspended in 1 ml of LB medium with 3 mM NaN₃. The procedure of centrifugation was repeated 3 times (3000 × g, 10 min, 4°C) to remove free phage particles. In the next step, 25 µl of phage-bacteria mixture was added to 25 ml of LB medium (time 0) and cultivated in an incubator shaker at 37°C. The number of infective centers was estimated from samples taken 5 min, 10 min, and 15 min after phage infection by plating under permissive conditions. Samples taken at the indicated times were cleared with chloroform and following the centrifugation step (2000 × g, 5 min, 4°C), the number of phage progeny was determined by using the double overlay plaque assay. Petri

dishes were incubated at 37°C for 20 h and then burst size was calculated as a ratio of phage titer to the number of infection centers.

Adsorption experiments

The rate of phage adsorption process was measured according to the procedure described by [30], with some modifications. Overnight cultures of the host strain were 100-fold diluted in LB medium supplemented with 10 mM MgSO₄ and 10 mM CaCl₂ and incubated with aeration at 37°C to an OD₆₀₀ of 0.2. Then, the phage stock solution was added to 6 x 10⁸ bacterial cells to an m.o.i. of 0.1. To determine the initial phage titer, the same volume of phage lysate was mixed with 3 ml of LB with 10 mM MgSO₄ and 10 mM CaCl₂ (time 0). At given time points, 100 µl samples were collected and added to 900 µl of TM buffer (10 mM Tris-HCl, 10 mM MgSO₄; pH 7.2). Following centrifugation (12000 x g, 1 min, 4°C), the obtained supernatant with unadsorbed phage particles was passed through the filter with a 0.22-µm-pore size SFCA membrane (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Ten-fold dilutions of the supernatant were prepared in TM buffer (10 mM Tris-HCl, 10 mM MgSO₄; pH 7.2) and full-plate titration was used to calculate the percentage of phage particles adsorbed on the host cell surface. For this purpose, a volume of 25 µl of each dilution was mixed with 1 ml of the overnight culture of the *E. coli* MG1655 strain and 2 ml of the top LB agar supplemented with 10 mM MgSO₄ and 10 mM CaCl₂. The mixture was gently vortexed for 10 s and poured onto the LB bottom agar containing the sublethal concentration of chloramphenicol (2.5 µg/ml; EPRO, Władysławowo, Poland). The percentage of the adsorbed phages was calculated as follows: ((initial titer—residual titer)/initial titer) x 100.

Bacterial RNA extraction

For the isolation of total RNA from host cells after infection with tested phage, the previously described procedure was employed [23]. Briefly, *E. coli* MG1655 bearing plasmid pUC18 or pUC18_24B_1 was grown in LB medium with shaking at 37°C to an OD₆₀₀ of 0.3. Then, 120 ml of the sample was centrifuged (2000 x g, 10 min, 4°C) and the obtained bacterial pellet was washed once with 30 ml of 0.85% NaCl (Chempur, Piekary Śląskie, Poland). Following centrifugation (2000 x g, 10 min, 4°C), the sample was suspended in 36 ml of the LB medium supplemented with 10 mM MgSO₄ and 10 mM CaCl₂. Phage particles were added to the bacterial culture to an m.o.i. of 1.5 and the mixture was incubated with aeration at 37°C. Samples were taken at times 0, 2, 6, and 8 min and treated with Killing buffer (200 mM NaN₃, 50 mM MgCl₂, 200 mM Tris-HCl pH 8.0) to inhibit the growth of host bacteria. The total RNA was extracted using the High Pure RNA Isolation Kit (Roche Diagnostics International, Rotkreuz, Switzerland) according to the manufacturer's protocol. To complete the digestion of genomic DNA from RNA samples, TURBO DNA-free™ Kit (Thermo Fischer Scientific, Waltham, Massachusetts, USA) was employed. The RNA concentration and quality were assessed by a NanoDrop spectrophotometer (Eppendorf, Hamburg, Germany) and agarose gel electrophoresis. Additionally, the absence of DNA was verified by PCR amplification and quantitative real-time reverse transcription-PCR (RT-qPCR) for all tested genes.

Reverse transcription

cDNA synthesis *via* reverse transcription was performed with 1.25 µg of RNA template, the Transcriptor Reverse Transcriptase, and random hexamer primers (Roche Diagnostics International, Rotkreuz, Switzerland), following the manual supplied by the provider.

Real-time PCR assay and data analysis

Quantification of transcripts in all tested samples was performed using LightCycler[®] 480 SYBR Green I Master (Roche Diagnostics International, Rotkreuz, Switzerland) according to the procedure described previously by [23]. The qPCR reaction contained 10 µl 2x SYBR Green I Master, 6.25 ng/µl cDNA, and 200 nM specific primers (Table 2). Real-time PCR amplification was performed with the following amplification program: 95°C for 5 min, followed by 55 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 15 s. The *icdA* was selected as a housekeeping gene that showed no changes in expression levels in the presence of tested bacteriophages [31]. No-template controls and a melting curve analysis were performed for each reaction to ensure the specificity of the product. The relative changes in gene expression were analyzed with LinRegPCR using the E-Method with efficiency correction [23, 29].

Metabolite extraction procedure

The extraction of intracellular metabolites from bacterial cells was performed according to the procedure described by [32], with some modifications. Briefly, an overnight culture of

Table 2. Oligonucleotides used in RT-qPCR assay.

Primer name	Sequence (5' → 3')
pF_Φ24B_xis	TATCGCGCCGGATGAGTAAG
pR_Φ24B_xis	CGCACAGCTTTGTATAATTGCG
pF_Φ24B_cIII	ATTCTTTGGGACTCCTGGCTG
pR_Φ24B_cIII	GTAAATTACGTGACGGATGGAAAC
pF_Φ24B_N	AGGCGTTTCGTGAGTACCTT
pR_Φ24B_N	TTACACCGCCCTACTCTAAGC
pF_Φ24B_cl	TGCTGTCTCCTTTCACACGA
pR_Φ24B_cl	GCGATGGGTGGCTCAAATT
pF_Φ24B_cro	CGAAGGCTTGTGGAGTTAGC
pR_Φ24B_cro	GTCTTAGGAGGAAGCCGTT
pF_Φ24B_cII	TGATCGCGCAGAACTGATTTAC
pR_Φ24B_cII	GACAGCCAATCATCTTTGCCA
pF_Φ24B_O	AAGCGAGTTTGCCACGAT
pR_Φ24B_O	GAACCCGAAGTCTTACCG
pF_Φ24B_Q	GGGAGTGAGGCTTGAGATGG
pR_Φ24B_Q	TACAGAGGTTCTCCCTCCCG
pF_Φ24B_cat	TCACCAGCTCACCCTCTTC
pR_Φ24B_cat	TTCTTGCCCGCCTGATGAAT
pF_Φ24B_S	CTGGGGAGTCTGCTGTTTGG
pR_Φ24B_S	GCCTTACGCCGGTCTTCTTT
pF_Φ24B_R	GGGTGGATGGTAAGCCTGT
pR_Φ24B_R	TAACCCGGTCGCATTTTTC
pF_Φ24B_d_ant	TGTTTGCTACCGGGCTGAAT
pR_Φ24B_d_ant	CCCTTGCCTGTATCAGCCA
pF_Φ24B_Rz	AACCGTGTTCTGTGTGTTGGT
pR_Φ24B_Rz	GGTTTGTGCCAGCCACAG
pF_Φ24B_Rz1	CGGATCAACGCCACCTGC
pR_Φ24B_Rz1	GTTGCATTATCCACGCCGG
pF_Φ24B_icdA	CGAAGCGGCTGACCTTAATTG
pR_Φ24B_icdA	GTTACGGTTTTTCGCGTTGAT

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bacterial cells was diluted 1:1000 in the fresh LB medium and cultivated at 37°C with shaking. At appropriate time points during growth, 16 units of OD₆₀₀ were harvested and centrifuged (7200 x g, 15 min, 4°C). The supernatant was removed, and the obtained pellet was subsequently washed three times with pre-chilled PBS (7200 x g, 15 min, 4°C; Thermo Fisher Scientific Inc., Waltham, MA, USA). Finally, the pellet was deeply frozen in liquid nitrogen and suspended in 1 ml of ultrapure water (Thermo Fisher Scientific Inc., Waltham, MA, USA). Bacterial cells were then disrupted by ultrasonication for 1 min at 20% power in an Omni-Ruptor 4000 (OMNI International, Kennesaw, USA) and centrifuged (13000 x g, 15 min, 4°C). The obtained cell lysate was filtered through Vivaspin centrifugal concentrator 3KDa (Merck, Darmstadt, Germany) and 448 µl of filtrate was mixed with 50 µl of DSS standard solution (Merck, Darmstadt, Germany) and 2 µl of D2O (Merck, Darmstadt, Germany). Each sample prepared in this way was transferred to a separate NMR tube for analysis.

NMR spectroscopy and quantification of metabolites

A series of 1D NMR spectra were acquired at 298 K on the 700 MHz Bruker Avance-III NMR spectrometer using the stock pulse sequence for a NOESY experiment with presaturation (*noesypr1d*). The combined acquisition delay and acquisition time was set to 5 s with a mixing time of 0.1 s. The final spectrum represented a total of 128 scans. Spectra were processed with minimal solvent suppression and 1.0 Hz exponential line broadening using NMRPipe [33]. Chenomx NMR Suite v9.02 (Chenomx) was used for all subsequent spectral analyses. The metabolites chosen for the survey were selected from the Chenomx 700 MHz database. To be included in the survey, a metabolite had to have at least one peak with an unambiguous chemical shift that could be accurately measured against a DSS standard with a precision of 0.005 ppm against the reference value. Metabolite plots were made with Pro-Fit v7.0.19 (Quantum Software).

Preparation of RNAs

A 20-nt 24B_1 RNA was chemically synthesized (Metabion, Planegg, Germany; S1 Table). For other RNAs, the DNA templates for the *in vitro* transcription were obtained by primer extension method (Metabion, Planegg, Germany; S2 Table) and transcribed with T7 RNA polymerase as previously described [34]. RNAs were purified by gel electrophoresis and 5'-³²P labeled using T4 polynucleotide kinase (Thermo Fisher Scientific Inc., Waltham, MA, USA), which was followed by phenol-chloroform extraction, purification on P-30 spin columns (Bio-Rad Laboratories Inc., Hercules, USA), and ethanol precipitation.

Protein overproduction and purification

The *E. coli* Hfq protein was purified as described elsewhere [35].

Equilibrium Binding Assay

Prior to use, RNAs were denatured by heating at 90°C during 2 min, then refolded on ice for 5 min. The desired range of unlabeled RNA or Hfq concentrations were obtained by serial dilution in the binding buffer (24 mM Tris, pH 7.5, 150 mM NaCl, 5% glycerol, 100 µg/ml BSA, 1 mM MgCl₂). Unlabeled RNAs or Hfq protein dilutions were then mixed in a 1:1 ratio with 2 nM ³²P-labeled RNAs in the binding buffer. The final concentrations were 1 nM of ³²P-RNA and 100 nM of unlabeled RNA or Hfq at maximum. Incubations were performed for 4 hours for RNA-RNA assays and for 0.5 hour for RNA-Hfq assays at RT in low-protein binding microplates. Afterward, 5 µl aliquots were loaded onto a 6% non-denaturing polyacrylamide

gel (19:1) and run in 0.5x TBE buffer. Gels were vacuum-dried, exposed to phosphor screens, and quantified using a phosphorimager (Fujifilm FLA-5000) with ImageQuant software. The equilibrium dissociation constant (K_d) values were calculated by fitting data to the quadratic equation using GraphPad Prism software. Standard deviations were calculated from at least three independent experiments.

Statistical analysis

The data are expressed as the means $\pm$ SD. All experiments were repeated at least three times. Comparisons of two average values were compared by using Student's *t*-test. Significant differences were marked by asterisks as follows: $p < 0.05$ (*), $p < 0.01$ (**), or $p < 0.001$ (***)

Results

Effects of the 24B_1 RNA overproduction on the bacteriophage development in *E. coli* bacteria

To test if the overproduction of the small, microRNA-size, 24B_1 molecule can influence the development of bacteriophage $\Phi 24_B$, we constructed an *E. coli* bacterial mutant bearing an additional copy of the gene coding for the 24B_1 molecule in the plasmid pUC18. After infection with wild-type phage $\Phi 24_B$, the production of the 24B_1 molecule occurred due to the activity of genes located in both the phage genome and plasmid pUC18_24B_1. Importantly, because of our observation that IPTG, the synthetic inducer of the *lac*-operon in pUC18, used at concentrations even lower than standard 1 mM, negatively affected the growth of *E. coli* bacteria used as hosts (S1 Fig), we decided to express the 24B_1 gene from its natural promoter P_{24B_1} .

To assess whether the specific aspects of phage development are targeted by the 24B_1 molecule under its overexpression conditions, we conducted a series of physiological experiments that provided results opposite to those obtained in the case of the phage deletion mutant $\Phi 24_B\Delta 24B_1$. This mutant was unable to produce the studied sRNA molecule as published by us previously [23]. The measurement of the efficiency of phage progeny production during the lytic cycle occurring after infection of non-lysogenic *E. coli* with the phage indicated that this process was less efficient in the bacterial strain bearing an additional copy of the 24B_1 gene on a plasmid relative to the control strain carrying the pUC18 vector (Fig 1).

Curiously, the adsorption efficiency of the $\Phi 24_B$ phage particles on the host cells MG1655 [pUC18_24B_1] bearing additional copies of the 24B_1 gene was higher relative to the control strain MG1655[pUC18] (Fig 2). This difference was significant at the initial minutes of the experiment before the phage entered subsequent phases of development in the bacterial cell.

When comparing the lysis profile of bacterial cultures MG1655[pUC18] and MG1655 [pUC18_24B_1] infected with $\Phi 24_B$ or $\Phi 24_B\Delta 24B_1$ at an m.o.i. = 1.5, we observed that the lysis efficiency was the lowest in the case of the bacterial variant with 24B_1 overproduction. In comparison with the culture lacking the 24B_1 gene (cells bearing pUC18 and infected with the phage deletion mutant $\Phi 24_B\Delta 24B_1$) the variant with 24B_1 overproduction showed significantly higher optical density OD₆₀₀ values (Fig 3A) and the numbers of survived cells (CFU/ml) (Fig 3B). Interestingly, a similar tendency between these two bacterial variants was revealed in the number of lysogens at 180 min after phage infection. Among cells that survived the infection, we noted 28% and 56% of lysogens of MG1655[pUC18]($\Phi 24_B\Delta 24B_1$) and MG1655[pUC18_24B_1]($\Phi 24_B$), respectively (Fig 3C).

The significant differences in the number of survived host bacteria MG1655[pUC18] and MG1655[pUC18_24B_1] as well as the number of lysogens appearing after the infection with

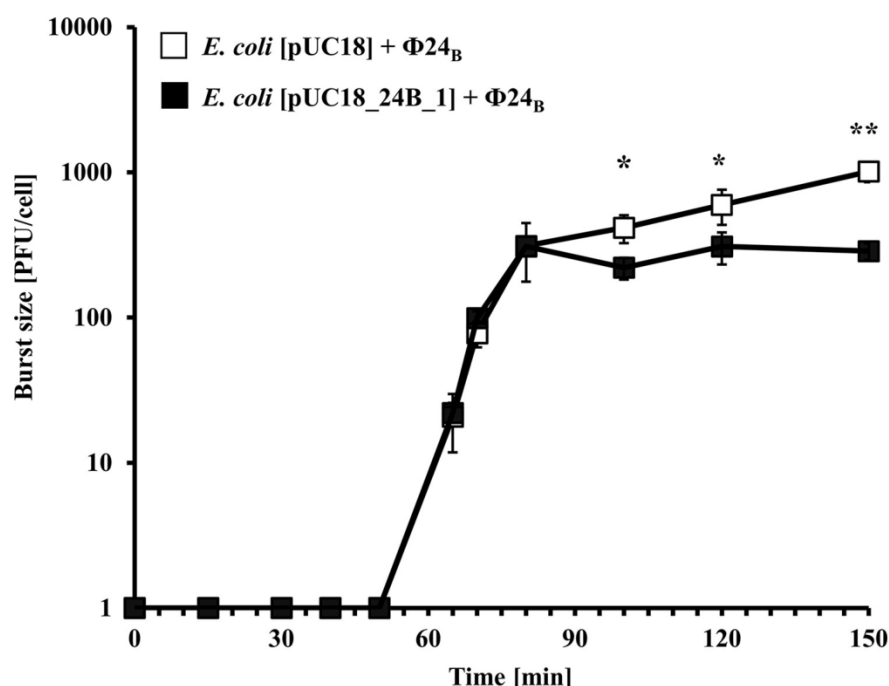


Fig 1. Kinetics of phage progeny production during the intracellular life cycle of Stx phage Φ24_B in the *E. coli* MG1655 host bearing pUC18 (□) or pUC18_24B_1 (■) plasmids. The presented results are mean values from three biological experiments with error bars indicating SD. Note that in some cases, the error bars are smaller than the sizes of symbols. Statistical analysis was performed by using a Student's *t*-test. The significance of differences between compared experimental variants is marked by asterisks: $p < 0.05$ (*) or $p < 0.01$ (**).

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phage Φ24_BΔ24B_1 and Φ24_B, respectively, were also observed at an m.o.i. = 5. These experiments were conducted under pro-lysogenic (poor in nutrients) and pro-lytic (rich in nutrients) conditions. The results obtained under pro-lysogenic conditions (Fig 4A and 4B) indicated that the number of survived bacterial cells (CFU/ml), and the number of lysogens among survivors were significantly higher in strains experiencing 24B_1 overproduction. The controls included MG1655 bacteria bearing vector pUC18 either (i) infected with wild-type phage Φ24_B, thus presenting the natural, unchanged level of 24B_1 expression, or (ii) infected with the phage deletion mutant Φ24_BΔ24B_1, thus having the 24B_1 production turned off completely. These results together demonstrate that lysogenization is favored by additional copies of the 24B_1 gene. Under pro-lytic conditions, the fraction of bacterial cells (CFU/ml) surviving the infection was also significantly higher when overproduction of 24B_1 occurred. It is worth noting that in this case, prophages were formed at similar frequencies in all tested variants (Fig 4C and 4D). Importantly, in relation to the control variants of cells non-treated with phages, the number of cells that survived the infection in all tested variants (MG1655 [pUC18] + Φ24_B; MG1655[pUC18_24B_1] + Φ24_B; and MG1655[pUC18] + Φ24_BΔ24B_1) was lower under rich, pro-lytic conditions (approximately 65, 80 and 20% of the not treated with phages controls, respectively) than under poor conditions (approximately 70, 115 and 50% of the controls, respectively). These observations suggest that under pro-lytic conditions the total number of the survived cells is generally lower than under pro-lysogenic conditions, and the majority of them (average 90%) are lysogens (Fig 4D).

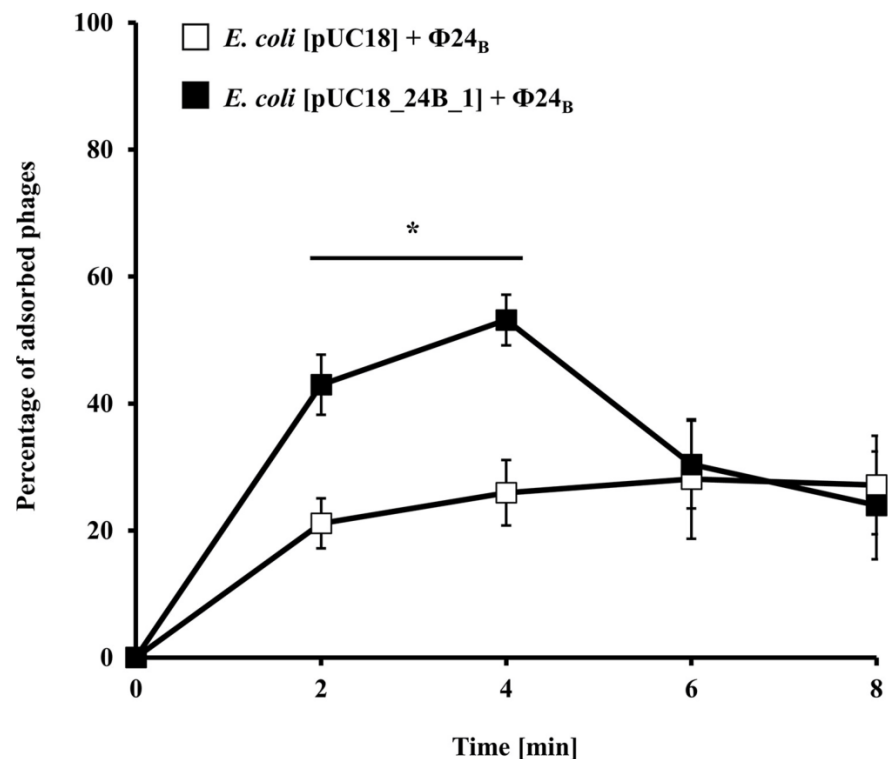


Fig 2. Kinetics of adsorption of Φ24_B phage on the surface of *E. coli* MG1655 bacterial cells bearing pUC18 (□) or pUC18_24B_1 (■) plasmids. The tested bacteriophage was added to the host suspensions to an m.o.i. of 0.1. The presented results are mean values from four biological experiments with error bars indicating SD. Statistical analysis was performed by using a Student's *t*-test. The significance of differences between compared experimental variants is marked by asterisks: $p < 0.05$ (*).

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Bacteriophage gene expression profiles in the presence of additional copies of the 24B_1 gene

A reverse transcription quantitative real-time PCR (RT-qPCR) assay was performed to measure levels of Φ24_B transcripts when 24B_1 was overproduced. At 2 min post-infection, some of the selected phage genes were expressed at similar levels in both hosts MG1655[pUC18] and MG1655[pUC18_24B_1]. Contrary to the initial time, drastic differences in levels of a few phage mRNAs were observed at later times. Within the interval of 6–8 min, expression of *cI*, *cII*, and *cIII* genes increased substantially when 24B_1 was overproduced relative to a control culture with a natural level of Φ24_B synthesis (Fig 5).

It is worth noting that all of the highly expressed genes are associated with the lysogenic pathway. *CI* is the main repressor of the phage lytic development that inhibits the expression of early phage genes from *p_R* and *p_L* promoters. *CII* stimulates the expression of the *cI* gene and the production of an integrase, another protein participating in the lysogenic cycle. Finally, *CIII* assists with the regulation of *CII* activity, being an inhibitor of the *CII*-degrading protease [3].

The 24B_1 sRNA—mediated metabolic changes in bacteria

We hypothesized that widespread changes in gene expression associated with 24B_1 would also be manifested by changes in bacterial metabolism. We began this investigation by

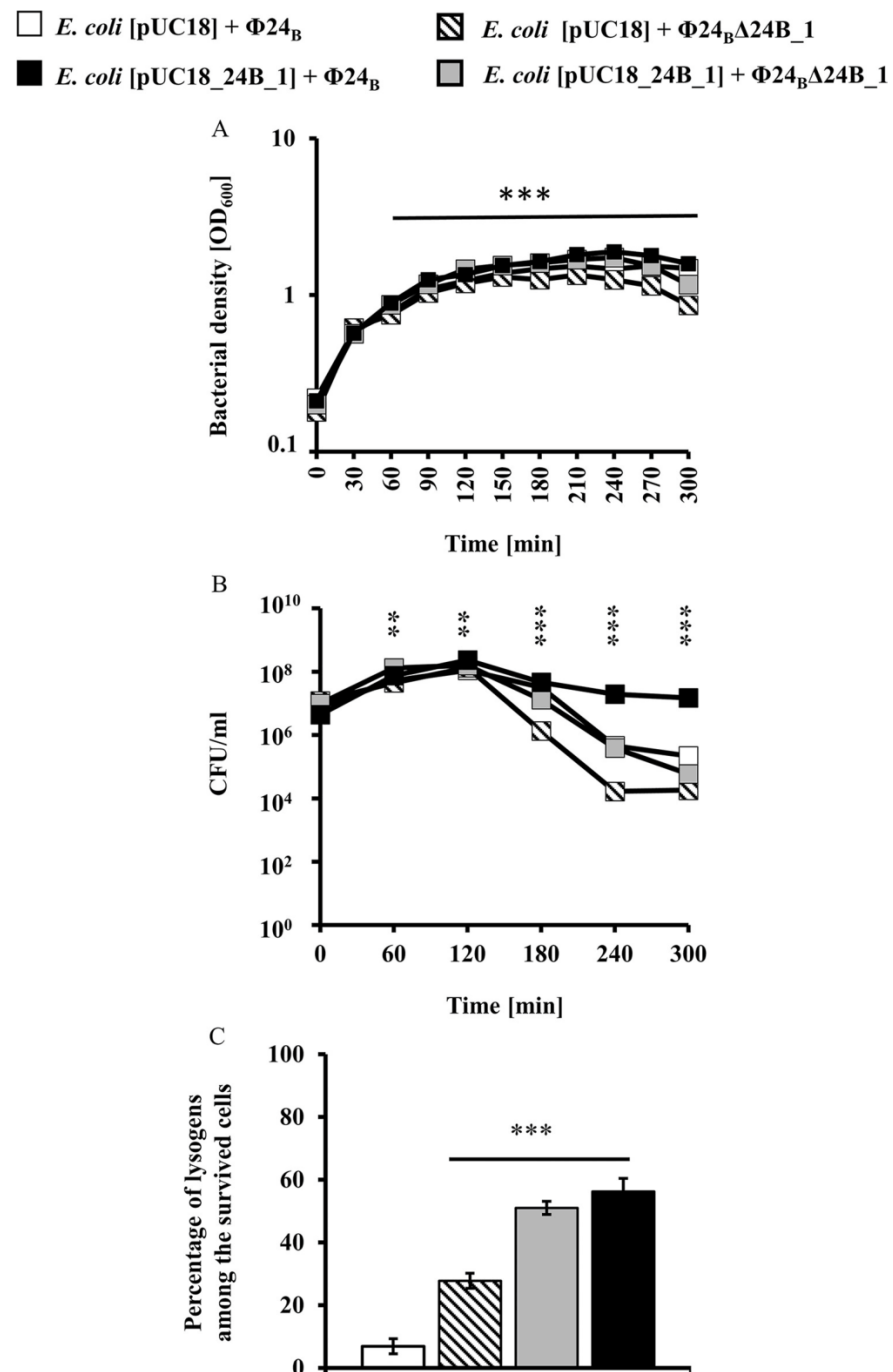


Fig 3. The lysis profile of host bacteria (a), their survivability presented as CFU/ml (b), and efficiency of creation of lysogens at 180 min. (c) after infection of *E. coli* MG1655 strain with Stx phages: $\Phi 24_B$ (squares $\square$ and $\blacksquare$) and $\Phi 24_B \Delta 24B_1$ (triangles $\triangle$ and $\blacktriangle$) at an m.o.i. of 1.5. Bacterial cells contained either the pUC18 (symbols $\square$ and $\blacksquare$) or pUC18_24B_1 (symbols $\triangle$ and $\blacktriangle$) plasmids. The presented results are mean values from three biological experiments with error bars indicating SD. Note that in some cases, the error bars are smaller than the sizes of symbols. Statistical analysis was performed by using a Student's *t*-test. The significance of differences between *E. coli* MG1655

[pUC18_24B_1] and *E. coli* MG1655[pUC18] infected with Φ 24_B or Φ 24_B Δ 24B_1, respectively, is marked by asterisks: $p < 0.01$ (**) or $p < 0.001$ (***).

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measuring the effects of 24B_1 on ATP levels. In comparison to variants lacking the 24B_1 sequence (MG1655[pUC18] and MG1655 strains), the 24B_1 molecule produced from its natural promoter p_{24B_1} (from both plasmid and phage genome) significantly decreased ATP levels in *E. coli* during the 4–6 hour interval of the experiment. Importantly, the bacterial growth rate and the number of live bacterial cells were similar in all tested variants thus excluding the possibility that the observed ATP level depends on the cell number (Fig 6).

Building upon the observation that ATP levels were different between variants producing 24B_1 molecule and those lacking its sequence, we measured a set of thirteen metabolites according to their unique chemical shifts in a 1D NMR spectrum (Fig 7). These metabolites could be categorized into aliphatic amino acids (alanine, threonine, valine, leucine, isoleucine), aromatic amino acids (phenylalanine, tyrosine), nucleobases (inosine, uracil, cytosine, guanosine), and energy production (NAD^+ , fumarate). The experiments were designed to sample metabolites at 4 hours and 6 hours of growth from a control MG1655 *E. coli* strain carrying an empty vector and an experimental strain with a plasmid supporting 24B_1 production. Unfortunately, a determination of ATP levels could not be accurately performed using the NMR method, because the adenine base chemical shifts at ~ 8.0 ppm could not be unambiguously assigned and integrated.

Comparing the 4-hour and 6-hour samples for the control plasmid in which no 24B_1 was overproduced, the metabolite levels were more or less stably maintained, especially for the most of amino acids. Over the period sampled, the metabolic profile of cultures overproducing 24B_1 experienced more changes. There was an excess of amino acids in the 4-hour sample that is diminished by the 6-hour sample, perhaps as the 24B_1 RNA continues to accumulate and the culture shifts from the exponential growth phase to the stationary phase.

NAD^+ and fumarate have unambiguous chemical shifts in all NMR spectra making these compounds useful for diagnostics of energy usage. A representative NMR spectrum is available in the Supporting information as S2 Fig. Within the citric acid cycle, NAD^+ is an electron carrier. Fumarate is the last substrate in the citric acid cycle to carry a double bond which is converted to malate upon the addition of water. Thus, if the efficiency or output of the citric acid cycle is diminished, there would be less fumarate available and an accumulation of NAD^+ that is not being converted to NADH throughout the cycle. The reciprocity between fumarate and NAD^+ appears to be enhanced when 24B_1 is overproduced.

The *sdhB* transcript as a target for the mature 24B_1 RNA molecule

Following our key observation that 24B_1 produced from its natural promoter p_{24B_1} significantly decreased the ATP levels in *E. coli*, we hypothesized that the citric acid cycle may be impaired. This hypothesis was further supported by the decrease in the levels of fumarate and increased levels of NAD^+ as determined by the NMR metabolomics study. Examining the idea that fumarate may be a key metabolite in addition to ATP, we compared the sequences of *sdhCDAB* genes that encode the individual subunits of succinate dehydrogenase Sdh. The sole membrane-bound Sdh enzyme participates in the citric acid cycle converting succinate to fumarate and takes part in the aerobic electron-transport pathway to generate energy via oxidative phosphorylation reactions. The bioinformatics analysis of the *sdhCDAB* genes sequences revealed the potential binding site for 24B_1 within the *sdhB* gene coding for the FeS subunit of succinate dehydrogenase. This binding site is located at the 5' end of the *sdhB* coding sequence between nucleotides 7–26 and was predicted by IntaRNA software [36] with

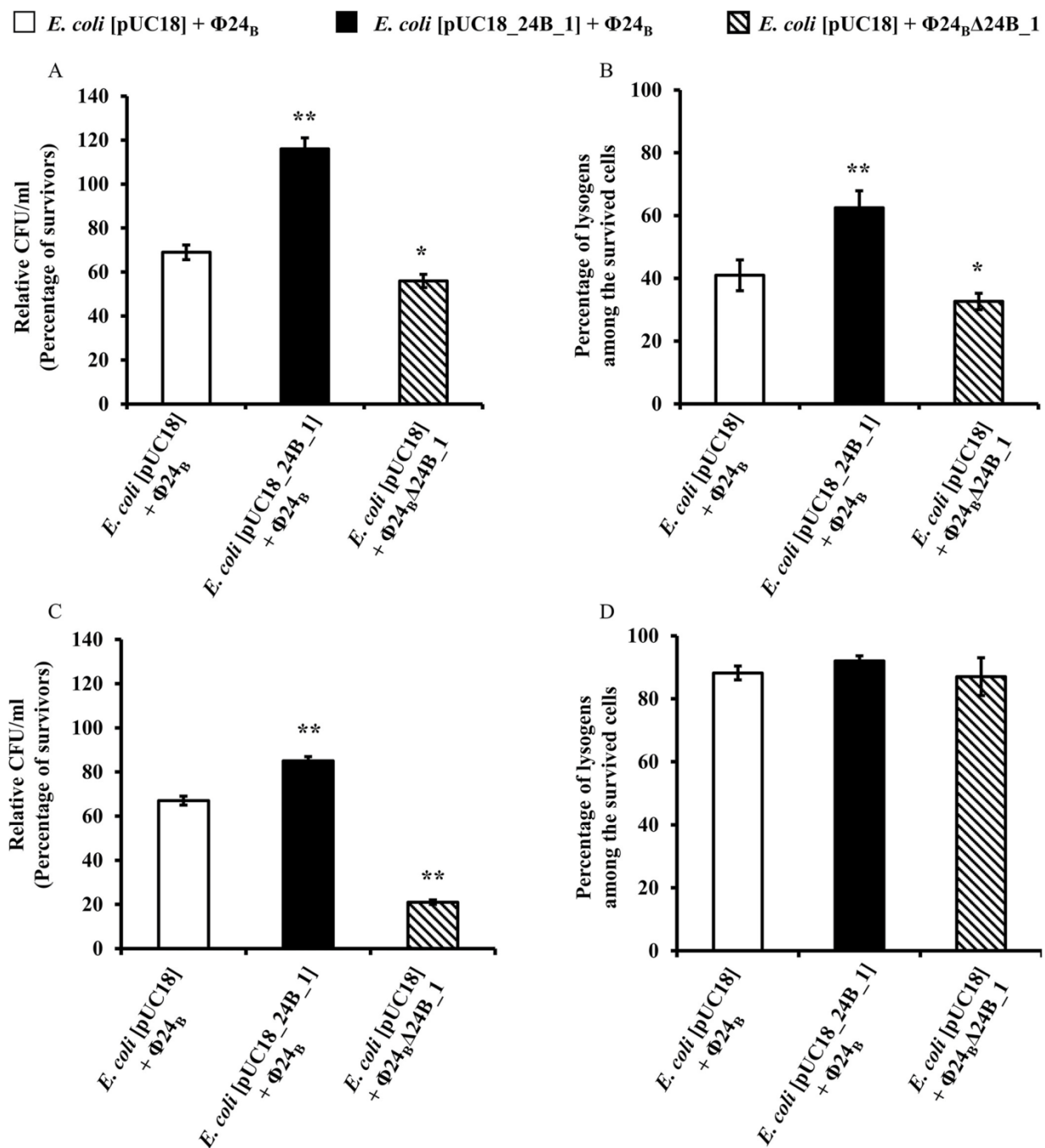


Fig 4. Survival of host bacteria (a, c) and efficiency of creation of lysogens (b, d) after infection of *E. coli* MG1655 strain bearing pUC18 or pUC18_24B_1 plasmids with Stx phages: Φ24_B and Φ24_BΔ24B_1 in pro-lysogenic (a, b) or pro-lytic (c, d) conditions. Tested bacteriophages were added to the host suspension to an m.o.i. of 5. Results are presented as mean values ± SD from three independent experiments. Statistical analysis was performed by using a Student's *t*-test. The significance of differences between compared experimental variants MG1655[pUC18_24B_1] + Φ24_B or MG1655[pUC18] + Φ24_BΔ24B_1 versus MG1655 [pUC18] + Φ24_B is marked by asterisks: *p* < 0.05 (*) or *p* < 0.01 (**).

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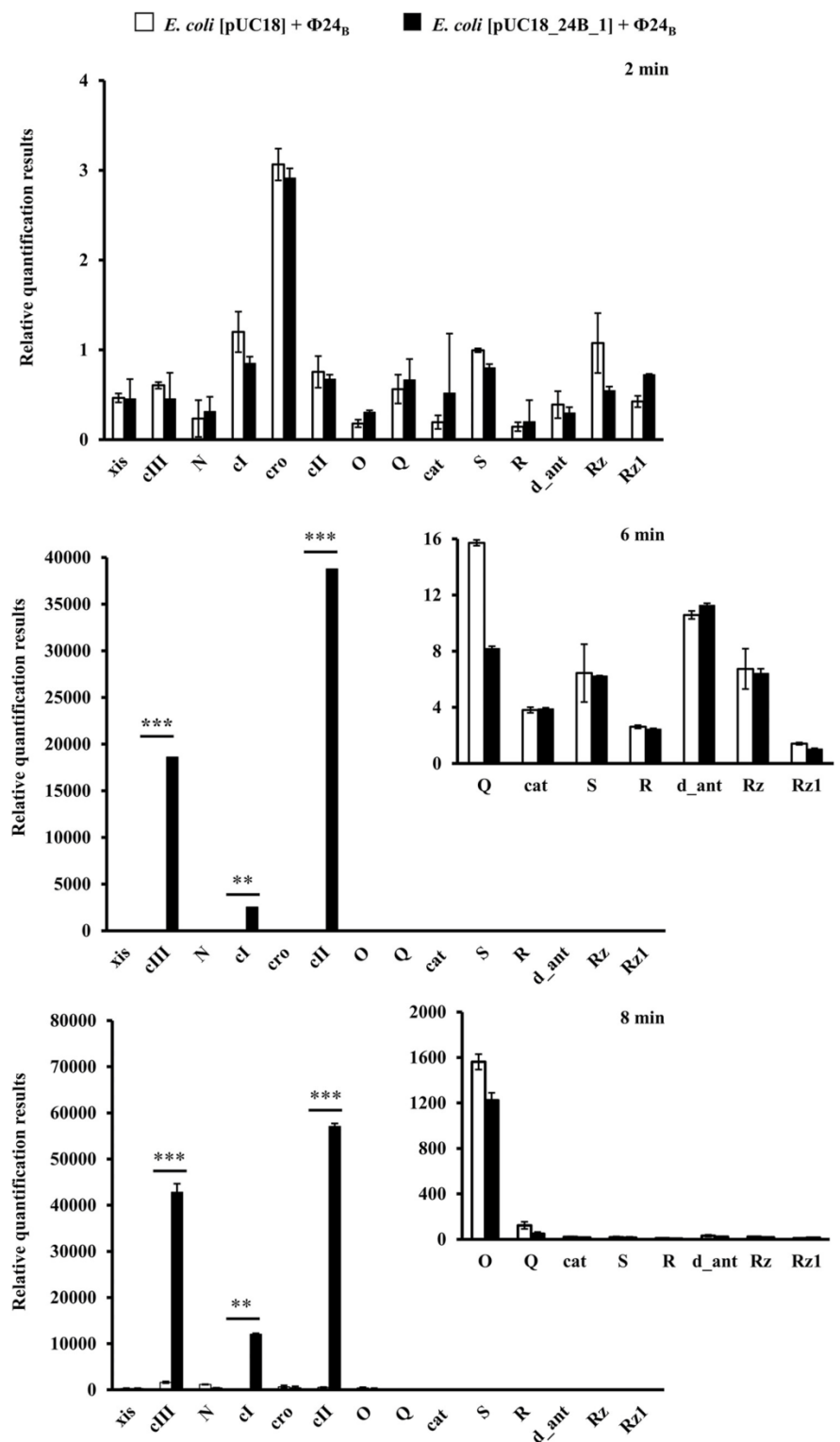


Fig 5. The expression levels of selected bacteriophage genes assessed by RT-qPCR after infection of host bacteria bearing pUC18 (□) or pUC18_24B_1 (■) plasmids with phage Φ 24_B at an m.o.i. of 1.5. The mRNA levels corresponding to particular genes were determined at the following times after infection: 2, 6, and 8 minutes. The presented results are mean values from three biological experiments with error bars indicating SD. Statistical analysis was performed by using Student's *t*-test. The significance of differences between compared experimental variants is marked by asterisks: $p < 0.01$ (**) or $p < 0.001$ (***).

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a high probability energy score (-10.56 kcal/mol) and hybridization energy value (-15.17 kcal/mol), as shown in S3 Fig. Importantly, only interaction energies below or equal to 0 are considered and such interactions are visualized by the IntaRNA program. A seed region typical for eukaryotic microRNAs was predicted by IntaRNA at positions 7–13 from the 5'-end of 24B_1 molecule. In eukaryotic cells, the seed sequence is essential for the binding of the microRNA to the target mRNA and has to be perfectly complementary.

To test whether mature 24B_1 or its precursor RNA can interact with the predicted pairing site at the 5' region of the coding sequence of *sdhB* gene we measured the binding of purified RNAs using an *in vitro* gelshift-based assay (Figs 8 and 9). The predicted structure of the 80-nt long precursor 24B_1 RNA showed that the sequence corresponding to the short 24B_1 is located in the apical portion of the long hairpin structure formed by the precursor RNA. This sequence is involved in a stable double-stranded structure, which would limit its availability for pairing to another sequence (Fig 8A). On the other hand, the 20-nt long mature 24B_1 RNA was predicted as less structured, although it still contained a short 4-basepair double helix (Fig 8B). Additionally, in this study we used a 56-nt long mRNA fragment including the untranslated region upstream of the *sdhB* mRNA and the beginning of its coding sequence. This mRNA fragment is predicted to form a secondary structure, in which the sequence complementary to 24B_1 is base-paired with a region including the Shine-Dalgarno sequence and the start codon of *sdhB* mRNA (Fig 8C).

When we compared the binding of the 24B_1 precursor and the mature 24B_1 to the 56-nt long fragment of the *sdhB* mRNA, we found that the mature 24B_1 molecule formed a complex with the *sdhB* mRNA, although the fraction bound at the maximum 100 nM mRNA concentration used was less than 10%, which did not allow calculating the binding affinity (Fig 9B and 9c). On the other hand, the binding of the 24B_1 precursor RNA to the *sdhB* mRNA fragment was not detected (Fig 9A). These data suggest that the mature 24B_1 RNA is more likely to interact with other RNA molecules than the precursor RNA because its structure is less stable and, hence, it is more available for pairing with other RNAs. In particular, these data show that the mature 24B_1 RNA can base-pair with the complementary sequence of the *sdhB* mRNA. The fact that the binding is not strong in the *in vitro* assay may indicate that the conformations of the interacting RNAs are more favorable for binding in the conditions *in vivo* or that other factors affect the RNA structure *in vivo*, thus influencing the binding.

Hfq binds the 24B_1 precursor form

To further characterize the properties of the mature 24B_1 RNA and its precursor, we compared their binding to *E. coli* Hfq protein, which is known to bind many small bacterial RNAs [38, 39]. Interestingly, the data showed that Hfq did bind to the precursor RNA with a K_d value of 25 nM. On the other hand, its binding to the mature 24B_1 RNA was not detected (Fig 10). The apical portion of the 24B_1 precursor RNA hairpin is stably base-paired, and, hence, would not be expected to bind this protein. However, the lower part of the structure, below the base-pair G₂₁-C₅₃, is likely less stable and contains an U-rich internal loop, which could potentially serve as a binding site for Hfq (Fig 8A). Overall, these data suggest that Hfq could affect the stability or processing of the precursor 24B_1 RNA, but not the mature 24B_1

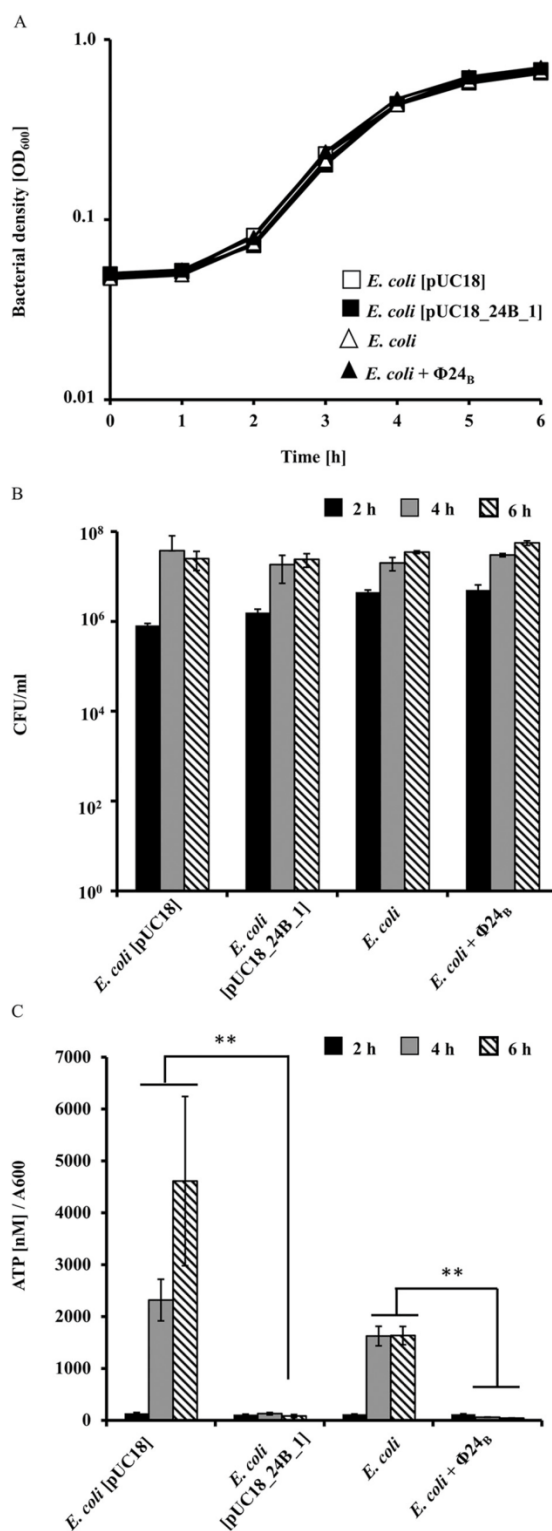


Fig 6. The bacterial growth kinetics (a), number of live bacterial cells presented as CFU/ml (b), and the ATP level (c) measured in *E. coli* control strains devoid of the 24B_1 molecule gene (MG1655 and MG1655[pUC18]), and in bacterial strains able to produce 24B_1 molecule form plasmid (MG1655[pUC18_24B_1]) or phage genome (MG1655 (Φ24_B)). Statistical analysis was performed by using a Student's *t*-test. The significance of differences between *E. coli* MG1655[pUC18] and *E. coli* MG1655 or *E. coli* MG1655[pUC18_24B_1] and *E. coli* MG1655 infected with Φ24_B are marked by asterisks: $p < 0.01$ (**).

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RNA. Hence, it is possible that mature 24B_1 RNA does not depend on RNA-binding proteins for its function, similar to *cis*-encoded antisense RNAs, which are not dependent on Hfq for pairing with their RNA targets [40].

Discussion

When considering the significance of small regulatory RNA molecules derived from phages, it becomes evident that they play a crucial role in the development of both the phage and its host [41]. In bacteria, these sRNAs have a profound impact on various processes such as virulence, colonization ability, motility, and cell growth or death [40]. Similarly, in the case of phages, they play vital roles during the early stages of infection, maintaining the state of lysogeny and suppressing the expression of late structural genes [42, 43].

The small RNA molecule, 24B_1, investigated in this work and originating from the Stx phage, deserves special attention in this context. While other microRNA-size, phage-encoded regulatory RNAs have not been described yet, similar molecules are well-documented in viruses that infect eukaryotic cells, particularly herpesviruses [44, 45]. Notably, this sRNA

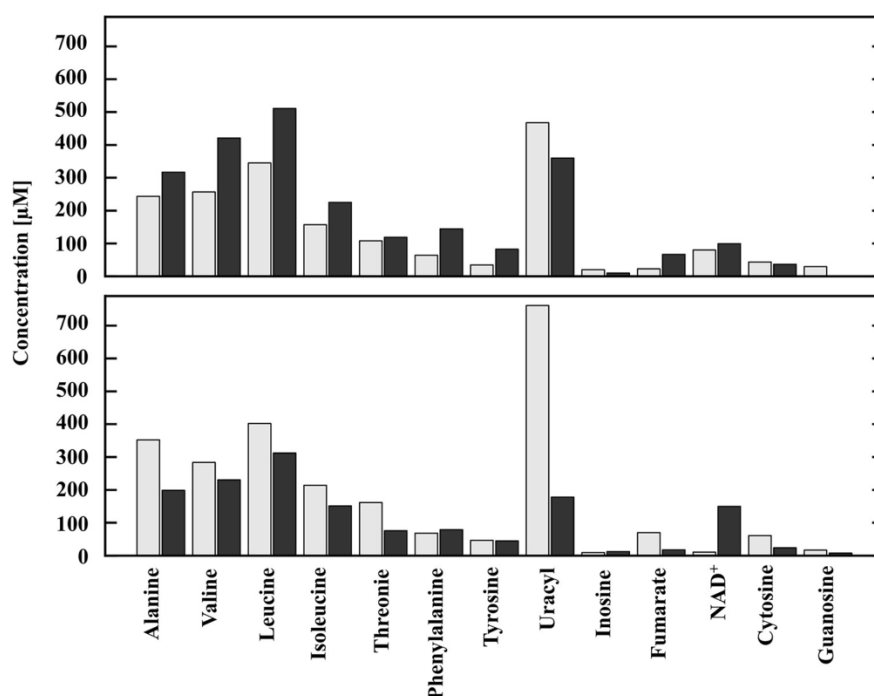


Fig 7. The metabolite profile of *E. coli* bacteria carrying pUC18 (light bars) and pUC18_24B_1 (dark bars) at four hours (upper panel) and six hours (lower panel) of the experiment. The results are the average of three repetitions and standard deviations do not exceed 10%.

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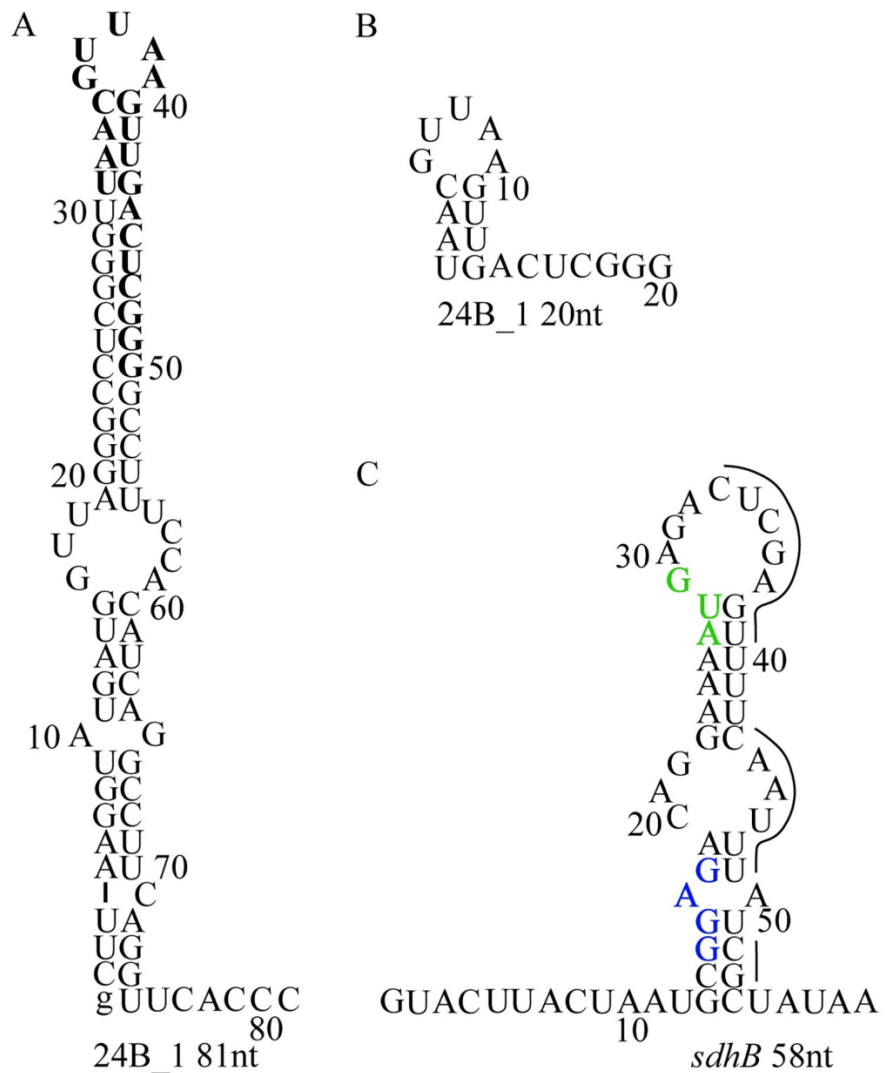


Fig 8. The secondary structures of the 81-nt long precursor of 24B_1 RNA (a), the mature 20-nt long 24B_1 RNA (b), and the sequence of the intergenic region and beginning of the coding sequence of *sdhB* gene (c), which were used in this study. The lower case g denotes guanosine residue added on the 5' end of 81-nt long 24B_1 RNA precursor to enable T7 RNA polymerase transcription. The sequence of the 20-nt long 24B_1 RNA within its precursor is marked in bold font (a). The Shine-Dalgarno sequence within the 5'-UTR of *sdhB* mRNA is marked in navy blue, the AUG start codon in green, and the sequence of *sdhB* mRNA predicted by IntaRNA as complementary to the mature 24B_1 RNA is marked with a line (c). The secondary structures were predicted using RNAstructure software [37].

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shares several similarities with herpesviral microRNAs. Importantly, like Stx phages, herpesviruses undergo two distinct stages in their lifecycle and are able to cause latent or lytic infections and switch between these states. Similar to phage lysogeny, viral latency represents a specialized form of persistent infection where most viral genes remain silenced. Herpesviruses can reactivate and transit to the lytic stage, leading to host cell lysis and the production of infectious viral progeny, which spreads to new cells [46].

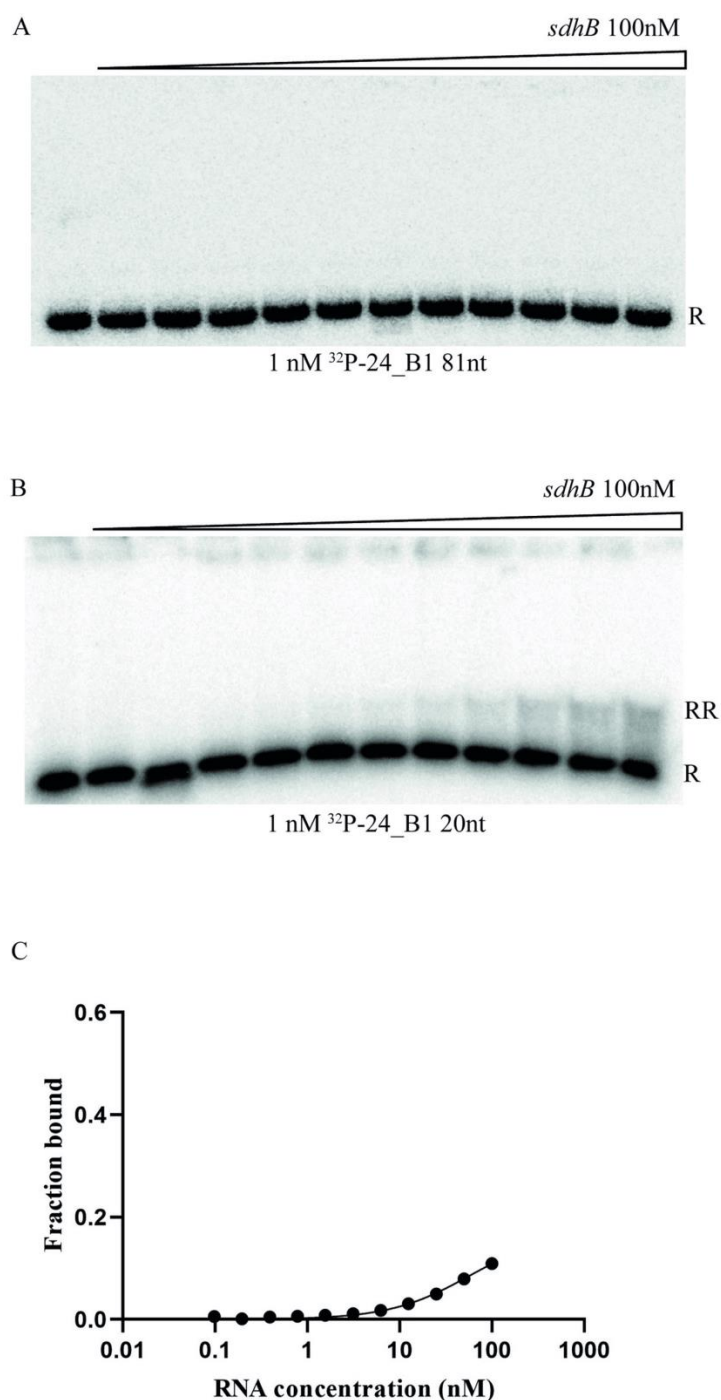


Fig 9. The mature 20-nt long 24B_1 RNA base-pairs with the 5'-UTR of *sdhB* mRNA. The pairing of the 32 P-labeled 81-nt long precursor of 24B_1 RNA (a) and mature 20-nt long 24B_1 RNA (b) to the 5'-UTR of *sdhB* mRNA was monitored using a gelshift assay. The fraction bound data from (b) were plotted versus RNA concentration in (c). Free 32 P-RNA is marked as R, and RNA-RNA complex as RR. The average fraction of mature 24B_1 RNA bound at the maximum 100 nM concentration of *sdhB* mRNA, calculated from three independent experiments, was $8 \pm 5\%$.

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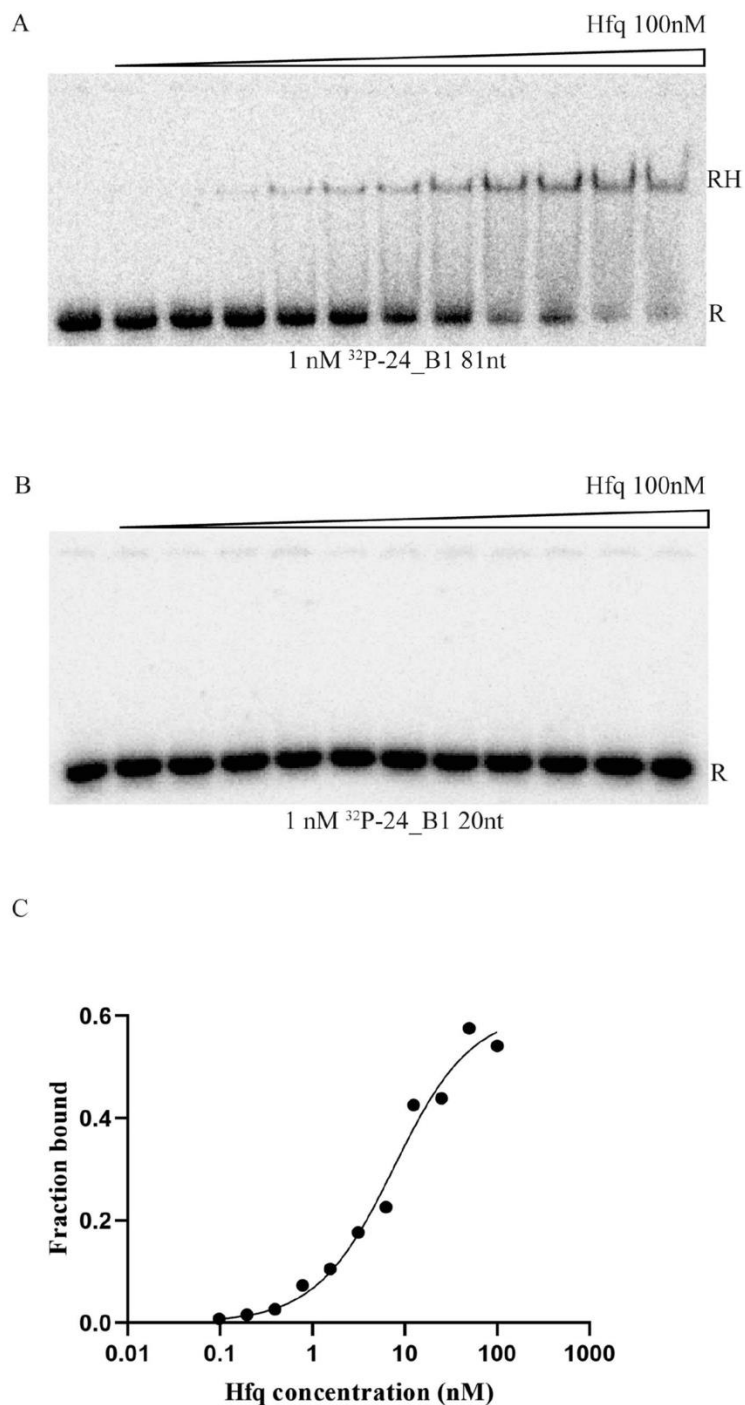


Fig 10. The Hfq protein binds to the 81-nt long precursor of 24B_1 RNA, but not to the mature 20-nt long 24B_1 RNA. The equilibrium binding of 32 P-labeled 81 nt-long precursor of 24B_1 (a), and the mature 20-nt long 24B_1 RNA (b) to the Hfq protein was monitored using a gelshift assay. Free 32 P-labeled RNAs are marked as R, and the RNA-Hfq complex as RH. The raw binding data from (a) were fit using the quadratic equation in (c). The average K_d value of 25 ± 13 nM was calculated from three independent experiments.

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Comparable to the identified herpesviral microRNAs, 24B_1 is a very short RNA molecule, only 20 nucleotides in length, produced through the cleavage of a precursor (80-nt long) transcript and likely undergoing a multi-step maturation process. Importantly, only the precursor form of 24B_1 binds the bacterial RNA-binding protein called Hfq (Fig 10). The functional roles of Hfq in sRNA action are fairly well understood and widely described in the literature [38, 39, 47, 48] however, we show for the first time that this protein is able to bind the precursor form of phage 24B_1 molecule. On the other hand, the performed analysis did not confirm the binding of the short (20-nt long) 24B_1 with Hfq protein. This result suggests that similar to microRNAs produced in eukaryotic cells, different proteins may participate at the particular stages of maturation and operation of such small RNA molecules in prokaryotic cells. Furthermore, like most identified herpesviral microRNAs, 24B_1 demonstrates limited complementarity with its identified target—the *sdhB* gene and actively promotes the phage's lysogenic state, which was confirmed under 24B_1 deletion and overproduction conditions (this work). Importantly, the imperfect pairing of the *SdhB* transcript to the 24B_1 seed region can be compensated by extended pairing interactions with the 3' end of the 24B_1 molecule, and this phenomenon is also quite frequently observed in the case microRNAs functioning in eukaryotic cells [49].

In herpesviruses, small regulatory RNAs classified as microRNAs have been identified as key players in the maintenance of viral latency [50]. The examples are miRNAs of the murine gammaherpesvirus-68 (MHV-68) which contribute to the maintenance of latent infection *in vivo*. The investigation of mutated viruses has unveiled that the presence of MHV-68 miRNAs are not necessary for the short-term replication of the virus, but they play a crucial role in establishing a persistent infection within memory B cells, ensuring a lifelong presence [51]. In turn, miRNAs of Kaposi's sarcoma-associated herpesvirus (KSHV), such as miR-K12-11 and miR-K12-3, prevent lytic reactivation by reducing the expression of cellular transcription factors MYB, C/EBP α , and Ets-1 [52, 53]. The prevailing model suggests that during latency, viral microRNAs are expressed at relatively high levels, facilitating this phase of infection and thus negatively impacting the expression of lytic genes. Otherwise, during the lytic cycle, the transcripts liberate from inhibition by these microRNAs and changes occur in their transcriptional activity. As a result, although most virus-encoded microRNAs are present and detectable during the lytic phase, their role becomes diminished during this stage of infection [54]. Interestingly, as described by us previously [23], the 24B_1 sRNA has been identified during phage lytic development, 80 minutes after prophage induction with mitomycin C. What is more, similar to viral microRNAs, the 24B_1 sRNA plays a significant role in maintaining the phage in the form of prophage. This is supported by previous results obtained for 24B_1 phage deletion mutant [23], and also under conditions of 24B_1 overexpression (this study). Here, we demonstrate that an excess of the 24B_1 molecule in the cell leads to a decrease in the efficiency of bacterial culture lysis after infection with Stx phage, and thus an increase in both the number of cells that survived the infection with phage, as well as the number of lysogens among survivors. The physiological effects were accompanied by increased levels of *cI*, *cII*, and *cIII* mRNAs which protein products are strongly associated with the lysogenic pathway. Importantly, this effect seems to be indirect as we did not find significant binding sites for the 24B_1 molecule within these genes. In this regard, the 24B_1 small RNA closely resembles eukaryotic microRNAs of viral origin.

In the context of persistent viral infections, the role of miRNAs encoded by herpesviruses is of utmost importance, influencing various aspects such as the metabolic processes in host cells [53]. Notably, miRNAs of Epstein-Barr virus (EBV) and KSHV disrupt the balance of metabolic processes through direct targeting of key proteins or indirect regulation of multiple signalling pathways. Recent research has shed light on the impact of several EBV miRNAs on

metabolic processes of cancer cells. For instance, the expression of miR-BART1 significantly upregulates the levels of phosphoglycerate dehydrogenase (PHGDH) in an NPC cell line called CNE1 [55]. PHGDH is a critical enzyme involved in the synthesis of serine and glycine, central metabolites that play essential roles in various biosynthetic pathways [56]. Regarding KSHV, it has been observed that viral miRNAs affect host cell energy metabolism through the regulation of multiple pathways. Firstly, KSHV miRNAs stabilize and activate the transcription factor HIF1 α —a master regulator of cell metabolism, by targeting EGLN2, a hypoxia-inducible factor prolyl hydroxylase. Secondly, KSHV miRNAs downregulate the mitochondrial heat shock protein A9 (HSPA9), which leads to a decrease in mitochondrial copy numbers and promotes anaerobic glycolysis (commonly known as the Warburg effect). The downregulation of EGLN2 and HSPA9 enables cancer cell proliferation even under low oxygen conditions [57]. In the field of oncology, the Warburg effect refers to the phenomenon observed in many cancer cells, where energy production primarily relies on anaerobic glycolysis rather than the typical citric acid cycle and oxidative phosphorylation occurring in the mitochondria of normal cells. This process involves high glucose uptake, followed by glycolysis and lactic acid fermentation in the cytosol, even in the presence of ample oxygen. While fermentation produces ATP with lower efficiency compared to the citric acid cycle and oxidative phosphorylation, it allows proliferating cells to utilize nutrients like glucose and glutamine more effectively for biomass production. Besides, it allows for bypassing the catabolic oxidation of nutrients into CO₂ and thus promotes anabolic processes [58, 59]. Surprisingly, the overproduced 24B_1 molecule seems to induce a similar effect in prokaryotic cells. As we have shown in this work, its overproduction caused a decrease in the level of ATP and fumarate, but stimulated an increase of NAD⁺, which is most likely not converted to NADH. This is most likely due to the negative regulation of the succinate dehydrogenase subunit gene (*sdhB*) by 24B_1 as *in vitro* binding of these two RNA transcripts has been observed. Sdh is the enzyme that participates in the citric acid cycle converting succinate to fumarate and takes part in the aerobic electron-transport pathway to generate energy *via* oxidative phosphorylation, thus is essential for the aerobic utilization of carbon sources. As reported recently, the Arg244His mutation in the amino acid sequence of the SDHB subunit attenuated ATP production and reduced mitochondrial number in *Caenorhabditis elegans*. These Arg244His SDHB mutants demonstrated elevated lactate/pyruvate levels, pointing to a missense-induced, Warburg-like aberrant glycolysis. Authors concluded that mutation in the amino acid sequence of SDHB rewires metabolism, resembling metabolic reprogramming in cancer [60]. Similar observations have been reported by other scientists who showed strong evidence that some mutations in SDH impair oxidative phosphorylation, which highly contributes to the Warburg metabolism switch observed in cancer cells [61–63]. The advantage of the Warburg phenomenon in cancer cells has been unclear however it was proposed that the metabolism of cancer cells, and indeed all proliferating cells, is adapted to facilitate the uptake and incorporation of nutrients into the biomass (e.g., nucleotides, amino acids, and lipids) needed to produce a new progeny of cells. Thanks to this, cancer cells metabolize nutrients in a manner conducive to proliferation rather than efficient ATP production [58]. Does the presence of the prophage in a bacterial cell cause a similar effect? Recent results obtained by Holt and collaborators [64] showed that integration of phage Φ 24_B into bacterial genome increases cell proliferation. When grown under normal conditions, both the single and double *E. coli* lysogens exhibited a considerable increase in cell numbers (exceeding 200%) compared to the MC1061 strain without Φ 24_B prophage [64]. Phage-mediated metabolic reprogramming of the bacterial cell was also presented by [65]. The authors showed that infection of the marine bacterium *Pseudoalteromonas* with PSA-HP1 phage alters central carbon and energy metabolisms. Their findings indicate that the glyoxylate shunt was

used in phage-infected cells instead of the classical citric acid cycle. Importantly, all *sdh* genes (*sdhABCD*) were downregulated 40 and 60 minutes after phage infection [65].

Undoubtedly energetic status is a significant modulator of lysogeny. The presence of a low ATP concentration within the intracellular environment creates a favorable condition for the accumulation of phage repressors of the lytic cycle. This condition not only promotes phage integration during new infections but also contributes to the persistence of existing prophages [66]. Host proteases, which rely on ATP for their proteolytic activity, serve as sensors of the cell's energy status and are regulated by cyclic AMP [67–69]. When intracellular ATP levels are high due to an abundant energy supply, the host proteases cleave the phage repressors expressed early in the infection, thus favoring the lytic cycle [68]. Thereby, the lysogeny dependence on the host's energetic metabolism and ATP amount is obvious. In this light, the observed pro-lysogenic role of the microRNA-size 24B_1 molecule takes on a new meaning. It seems that similar to herpesviral microRNAs, 24B_1 reprogrammes the host energy metabolism by altering the central carbon pathway and thus decreasing the cellular ATP level. The target site for the 24B_1 molecule appears to be the succinate dehydrogenase subunit gene *sdhB*, whose dysfunction may lead to disruption of the classical citric acid cycle and trigger the activation of an alternative pathway, characterized by the production of a smaller amount of ATP. In effect, the phage CI repressor is not cleaved by proteases and inhibits the phage lytic development in the bacterial cell thus maintaining the lysogeny state. This mechanism of action of the 24B_1 molecule on phage and host development seems to be highly probable in a light of the results presented in this work.

Conclusions

In many aspects (such as size, presence of the precursor form, and occurrence of a multi-step maturation process) the phage non-coding RNA molecule, named 24B_1, resembles herpesviral microRNAs. The 24B_1 molecule plays a significant role in maintaining the prophage state and reprogramming the host's energy metabolism. This is the first report demonstrating molecular mechanisms of functions of a microRNA-size RNA molecule of the phage origin.

Supporting information

S1 Fig. The growth kinetics of *E. coli* bacteria in the presence of 0.4 mM (●) and 0.8 mM (▲) concentrations of IPTG. Control *E. coli* bacteria (□) were cultured without the addition of IPTG.

(TIF)

S2 Fig. A representative NMR spectrum for metabolic changes in *E. coli* bacteria induced by the overproduced 24B_1 sRNA.

(TIF)

S3 Fig. The visualization of the potential interaction occurring between *SdhB* transcript and 24B_1 molecule performed by the IntaRNA program. The 24B_1 molecule is located at the bottom, whereas the target mRNA is at the top of the illustrated interaction. The energy score was calculated by the software.

(TIF)

S1 Table. Chemically synthesized RNA oligonucleotides.

(DOCX)

S2 Table. DNA oligonucleotides used to prepare templates for the *in vitro* transcription of the 81-nt long precursor of 24B_1 RNA and the 58-long fragment of 5'-UTR of *sdhB*

mRNA.

(DOCX)

S1 Raw images.

(PDF)

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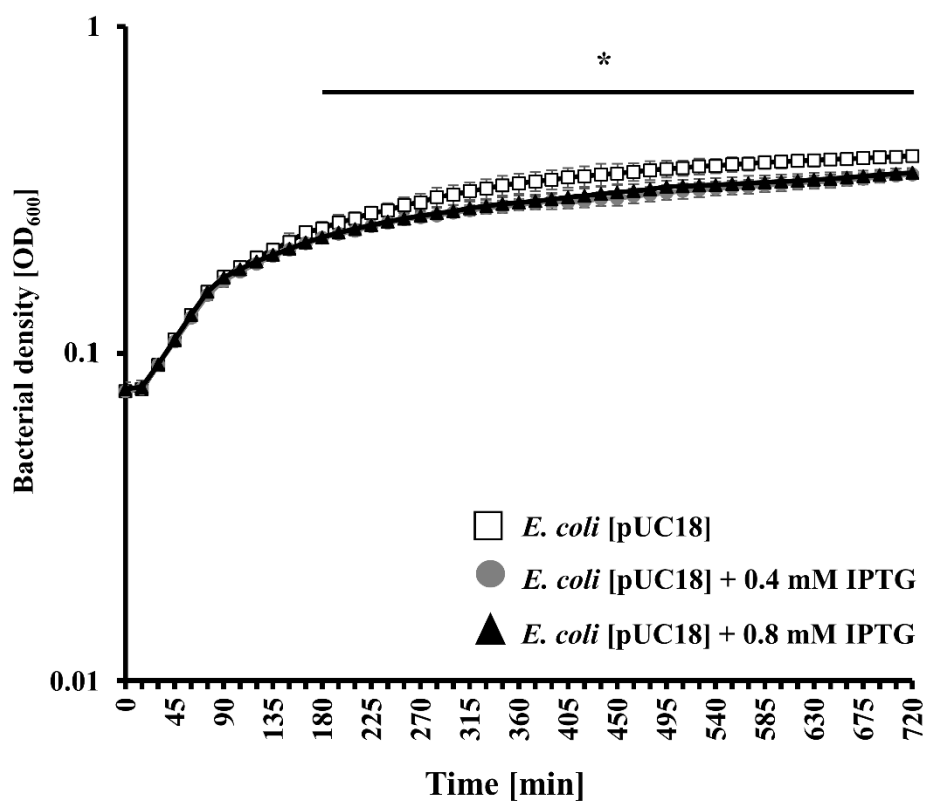
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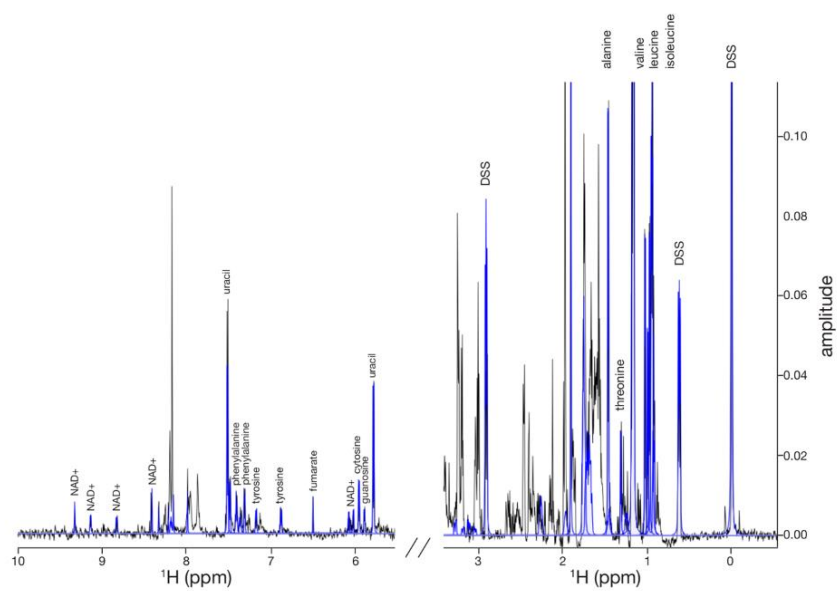
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Suplement do pracy nr 2

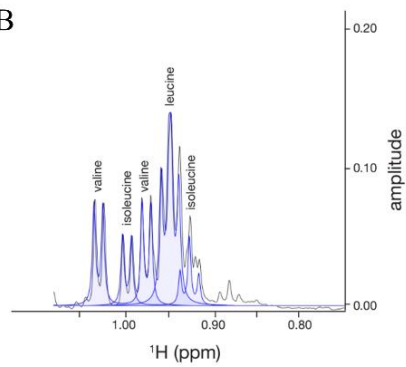


S1 Fig. The growth kinetics of *E. coli* bacteria in the presence of 0.4 mM (●) and 0.8 mM (▲) concentrations of IPTG.

A



B



S2 Fig. A representative NMR spectrum for metabolic changes in *E. coli* bacteria induced by the overproduced 24B_1 sRNA.

Diagram illustrating the formation of a 7-mer RNA hairpin structure:

Top strand (5' to 3'): 5' - AUGAGA UUU U CUAUA...UAA - 3'

Bottom strand (3' to 5'): 3' - GGGCUC AGUUGAAU GC AAU - 5'

Base pairing (indicated by vertical lines):

- Top strand (7): AUGAGA UUU U CUAUA...UAA
- Bottom strand (20): GGGCUC AGUUGAAU GC AAU

Numbering: 7 (top strand), 26 (top strand), 20 (bottom strand), 4 (bottom strand).

S3 Fig. The visualization of the potential interaction occurring between SdhB transcript and 24B-1 molecule performed by the IntaRNA program.

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Table S1. Chemically synthesized RNA oligonucleotides.

Name	Sequence (5' → 3')
24B_1 20	UAACGUUAAGUUGACUCGGG

Table S2. DNA oligonucleotides used to prepare templates for the *in vitro* transcription of the 81-nt long precursor of 24B_1 RNA and the 58-long fragment of 5'-UTR of *sdhB* mRNA.

Name	Sequence (5' → 3')
24_B1 81_F	TAATACGACTCACTATAGCTTAAGGTATGATGGTTAGGGCCTCG GGTTAACGTTAAGTTGAC TCGG
24_B1 81_R	GGGTGAACCTGAAGGCCTGATGTGGAAAGGCCCGAGTCAAC TTAACGTTAACC
sdhB_F	TAATACGACTCACTATAGTACTTACTAATGCGGAGACAGGAAA ATGAGACTCG
sdhB_R	TTATAGCGATAAATTGAAAACCTCGAGTCTCATTTTCCTGTCTCC

Fig. 9 A

The image was obtained using a phosphorimager (Fujifilm FLA-5000) with ImageQuant software.

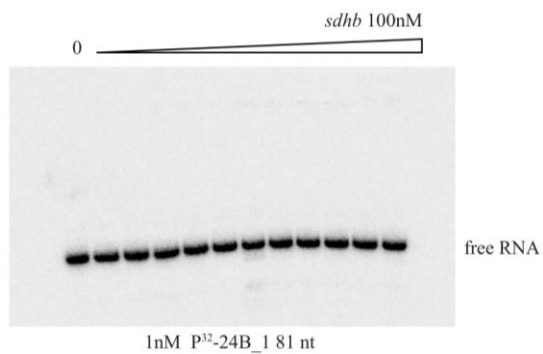


Fig. 9 B

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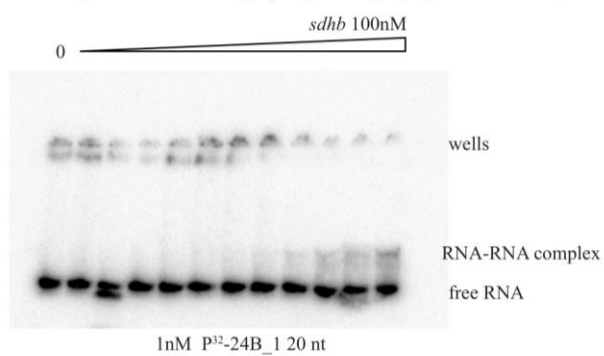


Fig. 10 A

The image was obtained using a phosphorimager (Fujifilm FLA-5000) with ImageQuant software.

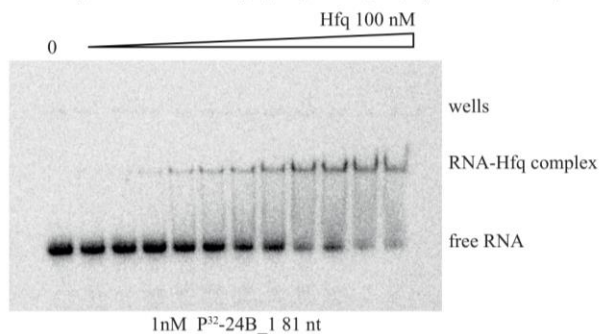
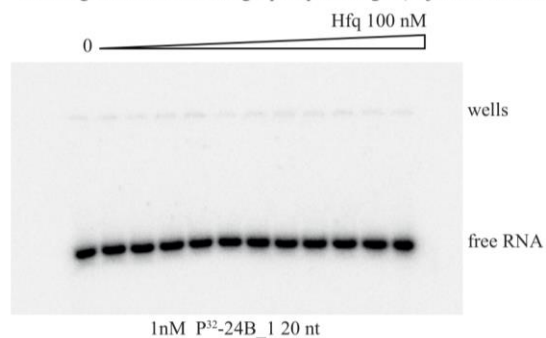


Fig. 10 B

The image was obtained using a phosphorimager (Fujifilm FLA-5000) with ImageQuant software.



Supplementary raw gel images for figures 9 and 10.

Oświadczenia współautorów

Sylvia Bloch

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IMIĘ I NAZWISKO

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DATA

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AFILIACJA

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Niniejszym oświadczam, iż mój wkład w publikację:

BLOCH, S., LEWANDOWSKA, N., ZWOLENKIEWICZ, J., MACH, P., LUKASIAK, A., OLEJNICZAK, M., DONALDSON, L. W., WĘGRZYN, G., & NEJMAN-FALEŃCZYK, B. (2023). BACTERIOPHAGE-ENCODED 24B_1 MOLECULE RESEMBLES HERPESVIRAL MICRORNAS AND PLAYS A CRUCIAL ROLE IN THE DEVELOPMENT OF BOTH THE VIRUS AND ITS HOST. PLOS ONE, 18(12), E0296038. [HTTPS://DOI.ORG/10.1371/JOURNAL.PONE.0296038](https://doi.org/10.1371/JOURNAL.PONE.0296038)

polegał na: (i) przeprowadzeniu eksperymentu mającego na celu analizę wewnątrzkomórkowego rozwoju faga $\Phi 24_B$ podczas jednego cyklu infekcyjnego w warunkach nadekspresji cząsteczki 24B_1; (ii) określeniu kinetyki lizy komórek gospodarza niosących plazmid pUC18 lub pUC18_24B_1 po infekcji fagiem $\Phi 24_B$ lub $\Phi 24_B\Delta 24B_1$ (pomiar OD_{600} , wyznaczenie wartości CFU/ml oraz PFU/ml, oszacowanie wydajności lizogenizacji komórek gospodarza); (iii) zbadaniu przeżywalności komórek gospodarza oraz określenie liczby lizogenów po infekcji bakterii *Escherichia coli* niosących plazmid pUC18 lub pUC18_24B_1 fagiem $\Phi 24_B$ lub $\Phi 24_B\Delta 24B_1$ w warunkach sprzyjających rozwojowi litycznemu lub lizogenicznemu wirusa; (iv) określeniu zmian w gęstości optycznej hodowli bakteryjnych (OD_{600}), w wartościach CFU/ml oraz w poziomach ATP podczas wzrostu bakterii *E. coli*, *E. coli* [pUC18], *E. coli* [pUC18_24B_1] oraz po infekcji bakterii *E. coli* fagiem $\Phi 24_B$; (v) analizie poziomu ekspresji genów z wykorzystaniem techniki RT-qPCR przeprowadzonej w warunkach nadekspresji cząsteczki 24B_1 podczas procesu infekcji bakterii *E. coli* bakteriofagiem $\Phi 24_B$; (vi) współpracowaniu metody ekstrakcji metabolitów z komórek bakteryjnych oraz współudziale w przygotowaniu prób do analizy NMR; (vii) współudziale w planowaniu doświadczeń i interpretacji uzyskanych wyników; (viii) przygotowaniu rycin; (ix) współudziale w przygotowaniu i redagowaniu manuskryptu; (x) pomocy w przygotowaniu odpowiedzi na uwagi recenzentów.

Sylvia Bloch

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PODPIS

Natalia Lewandowska

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IMIĘ I NAZWISKO

g. 04. 2026

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DATA

Katedra Biologii Molekularnej
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Uniwersytet Gdański

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AFILIACJA

OŚWIADCZENIE

Niniejszym oświadczam, iż mój wkład w publikację:

BLOCH, S., LEWANDOWSKA, N., ZWOLENKIEWICZ, J., MACH, P., ŁUKASIAK, A., OLEJNICZAK, M., DONALDSON, L. W., WĘGRZYN, G., & NEJMAN-FALEŃCZYK, B. (2023). BACTERIOPHAGE-ENCODED 24B_1 MOLECULE RESEMBLES HERPESVIRAL MICRORNAS AND PLAYS A CRUCIAL ROLE IN THE DEVELOPMENT OF BOTH THE VIRUS AND ITS HOST. PLOS ONE, 18(12), E0296038. [HTTPS://DOI.ORG/10.1371/JOURNAL.PONE.0296038](https://doi.org/10.1371/JOURNAL.PONE.0296038)

polegał na: (i) udziale w przeprowadzeniu eksperymentu mającego na celu analizę wydajności adsorpcji; (ii) wykonaniu analizy bioinformatycznej wiązania mRNA genu *sdhB* z cząsteczką sRNA 24B_1 i potwierdzenie tego wiązania w teście wiązania RNA za pomocą metody różnicowej migracji kompleksów w żelu poliakrylamidowym; (iii) udziale w identyfikacji wiązania długiej, prekursorowej formy cząsteczki 24B_1 z białkiem Hfq; (iv) udziale w opracowaniu metod badawczych, planowaniu doświadczeń oraz w analizie i interpretacji wyników; (v) napisaniu wstępu i współtworzeniu rozdziału poświęconego opisowi materiałów i metod; (vi) przygotowaniu rycin z wyników przeprowadzonych eksperymentów; (vii) współudziale w przygotowaniu i redagowaniu manuskryptu; (viii) pomocy w przygotowaniu odpowiedzi dla recenzentów.

Natalia Lewandowska

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PODPIS

Joanna Zwolenkiewicz-Nowak

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IMIĘ I NAZWISKO

31.03.2020

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DATA

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Wydział Biologii
Uniwersytet Adama Mickiewicza w Poznaniu

.....
AFILIACJA

OŚWIADCZENIE

Niniejszym oświadczam, iż mój wkład w publikację:

BLOCH, S., LEWANDOWSKA, N., ZWOLENKIEWICZ, J., MACH, P., ŁUKASIAK, A.,
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PLOS ONE, 18(12), E0296038. [HTTPS://DOI.ORG/10.1371/JOURNAL.PONE.0296038](https://doi.org/10.1371/JOURNAL.PONE.0296038)

polegał na: współudziale w przeprowadzaniu eksperymentów oraz analizie i opisanu danych w zakresie
wiązań RNA za pomocą metody różnicowej migracji kompleksów w żelu poliakrylamidowym, jak i
przygotowaniu figur z tego zakresu.

Joanna Zwolenkiewicz-Nowak

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PODPIS

Paulina Mach

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IMIĘ I NAZWISKO

27.03.2026

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DATA

Katedra Biologii Molekularnej
Wydział Biologii
Uniwersytet Gdański

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AFILIACJA

OŚWIADCZENIE

Niniejszym oświadczam, iż mój wkład w publikację:

BLOCH, S., LEWANDOWSKA, N., ZWOLENKIEWICZ, J., MACH, P., ŁUKASIAK, A.,
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PLOS ONE, 18(12), E0296038. [HTTPS://DOI.ORG/10.1371/JOURNAL.PONE.0296038](https://doi.org/10.1371/JOURNAL.PONE.0296038)

polegał na zbadaniu wpływu IPTG na wzrost hodowli bakteryjnej *E. coli*.

Paulina Mach

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PODPIS

Aleksandra Łukasiak

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IMIĘ I NAZWISKO

08.04.2016

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DATA

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Wydział Biologii
Uniwersytet Gdański

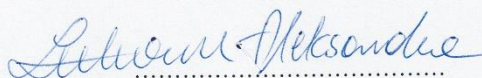
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AFILIACJA

OŚWIADCZENIE

Niniejszym oświadczam, iż mój wkład w publikację:

BLOCH, S., LEWANDOWSKA, N., ZWOLENKIEWICZ, J., MACH, P., ŁUKASIAK, A.,
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PLOS ONE, 18(12), E0296038. [HTTPS://DOI.ORG/10.1371/JOURNAL.PONE.0296038](https://doi.org/10.1371/JOURNAL.PONE.0296038)

polegał na wykonaniu wstępnych eksperymentów mających na celu analizę wydajności adsorpcji
oraz udziale w optymalizacji tej metody.



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PODPIS

Mikołaj Olejniczak

24.03.2026

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IMIĘ I NAZWISKO

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DATA

Instytut Biologii Molekularnej i Biotechnologii
Wydział Biologii
Uniwersytet im. Adama Mickiewicza w Poznaniu

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AFILIACJA

OŚWIADCZENIE

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BLOCH, S., LEWANDOWSKA, N., ZWOLENKIEWICZ, J., MACH, P., ŁUKASIAK, A.,
OLEJNICZAK, M., DONALDSON, L. W., WĘGRZYN, G., & NEJMAN-FALEŃCZYK, B. (2023).
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PLOS ONE, 18(12), E0296038. [HTTPS://DOI.ORG/10.1371/JOURNAL.PONE.0296038](https://doi.org/10.1371/JOURNAL.PONE.0296038)

polegał na: współudziale w zaplanowaniu eksperymentów oraz analizie danych w zakresie wiązania
RNA za pomocą metody różnicowej migracji kompleksów w żelu poliakrylamidowym, jak również w
opisanu otrzymanych tą drogą wyników.



Signed by /
Podpisano przez:

Mikołaj Olejniczak

Date / Data:
2026-03-24 12:21

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PODPIS



Raport z weryfikacji podpisu

INFORMACJE O DOKUMENCIE:	
Nazwa pliku	Olejniczak M_1-sig.pdf
Data weryfikacji podpisów	2026-04-09 12:27:56

SZCZEGÓŁY WERYFIKACJI:

Mikołaj Olejniczak	24.03.2026
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Instytut Biologii Molekularnej i Biotechnologii	
Wydział Biologii	
Uniwersytet im. Adama Mickiewicza w Poznaniu	
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AFILIACJA	
OŚWIADCZENIE	
Niniejszym oświadczam, iż mój wkład w publikację:	
BLOCH, S., LEWANDOWSKA, N., ZWOLENKIEWICZ, J., MACH, P., LUKASIAK, A., OLEJNICZAK, M., DONALDSON, L. W., WĘGRZYN, G., & NEJMAN-FALEŃCZYK, B. (2023). BACTERIOPHAGE-ENCODED 24B_1 MOLECULE RESEMBLES HERPESVIRAL MICRORNAS AND PLAYS A CRUCIAL ROLE IN THE DEVELOPMENT OF BOTH THE VIRUS AND ITS HOST. PLOS ONE, 18(12), E0296038. HTTPS://DOI.ORG/10.1371/JOURNAL.PONE.0296038 polegał na: współudziale w zaplanowaniu eksperymentów oraz analizie danych w zakresie wiązania RNA za pomocą metody różnicowej migracji kompleksów w żelu poliakrylamidowym, jak również w opisanu otrzymanych tą drogą wyników.	
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Podpis: Pozytywny	
Rodzaj certyfikatu	Kwalifikowany
Format podpisu	PADES-BES
Data złożenia podpisu	2026-03-24 12:21:08
Podpis zawiera znacznik czasu	Brak
Certyfikat podpisującego / składającego pieczęć	Nazwa powszechna: Mikołaj Olejniczak Kraj: PL
Numer seryjny certyfikatu	405870549103394775824420180675796712102703250001
Wystawca certyfikatu	Nazwa powszechna: COPE SZAFIR - Kwalifikowany Nazwa organizacji: Krajowa Izba Rozliczeniowa S.A. Kraj: PL Identyfikator organizacji: VATPL-5260300517
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	Kwalifikowany podpis poprawnie zweryfikowany certyfikatem kwalifikowanym
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Prof. Logan W. Donaldson

Toronto,

.....
FIRST NAME AND LAST NAME

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DATE

York University
Department of Biology

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AFFILIATION

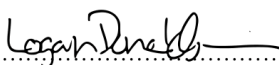
STATEMENT

I hereby declare that my contribution to the publication:

BLOCH, S., LEWANDOWSKA, N., ZWOLENKIEWICZ, J., MACH, P., ŁUKASIAK, A., OLEJNICZAK, M., DONALDSON, L. W., WĘGRZYN, G., & NEJMAN-FALEŃCZYK, B. (2023). BACTERIOPHAGE-ENCODED 24B_1 MOLECULE RESEMBLES HERPESVIRAL MICRORNAS AND PLAYS A CRUCIAL ROLE IN THE DEVELOPMENT OF BOTH THE VIRUS AND ITS HOST. *PLOS ONE*, 18(12), e0296038. [HTTPS://DOI.ORG/10.1371/JOURNAL.PONE.0296038](https://doi.org/10.1371/JOURNAL.PONE.0296038)

included the participation in: (i) planning and execution of experiments based on the quantification of metabolites by NMR spectroscopy, (ii) manuscript writing, (iii) funding acquisition, and (iv) results discussions.

SIGN

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SIGNATURE

Natalia Lewandowska

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AFILIACJA

OŚWIADCZENIE

Niniejszym oświadczam, iż dokument określający wkład prof. Logana Donaldsona w publikację:

BLOCH, S., LEWANDOWSKA, N., ZWOLENKIEWICZ, J., MACH, P., ŁUKASIAK, A., OLEJNICZAK, M., DONALDSON, L. W., WĘGRZYN, G., & NEJMAN-FALEŃCZYK, B. (2023). BACTERIOPHAGE-ENCODED 24B_1 MOLECULE RESEMBLES HERPESVIRAL MICRORNAS AND PLAYS A CRUCIAL ROLE IN THE DEVELOPMENT OF BOTH THE VIRUS AND ITS HOST. PLOS ONE, 18(12), E0296038. [HTTPS://DOI.ORG/10.1371/JOURNAL.PONE.0296038](https://doi.org/10.1371/JOURNAL.PONE.0296038)

z przyczyn niezależnych od osoby ubiegającej się o nadanie stopnia doktora, wpłynął jedynie w wersji elektronicznej (skan dokumentu). Jednocześnie oświadczam, że zadeklarowany wkład został ujęty w oświadczeniu autora korespondencyjnego, a treść skanu jest w pełni zgodna z wolą współautora.

Natalia Lewandowska
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PODPIS

Bożena Nejman-Faleńczyk

24.04.2026 r.

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IMIĘ I NAZWISKO

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DATA

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Wydział Biologii
Uniwersytet Gdański

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AFILIACJA

OŚWIADCZENIE

Niniejszym oświadczam, iż wkład prof. Logana Donaldsona w publikację:

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PLOS ONE, 18(12), E0296038. [HTTPS://DOI.ORG/10.1371/JOURNAL.PONE.0296038](https://doi.org/10.1371/JOURNAL.PONE.0296038)

polegał na współudziale w: (i) planowaniu i wykonaniu eksperymentów opartych na
ilościowym oznaczaniu metabolitów metodą spektroskopii NMR, (ii) pisaniu manuskryptu, (iii)
pozyskaniu funduszy oraz (iv) dyskusji wyników.

B. Nejman-Faleńczyk
.....

PODPIS

Grzegorz Węgrzyn

21 marca 2026 r.

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IMIĘ I NAZWISKO

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DATA

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Uniwersytet Gdański

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AFILIACJA

OŚWIADCZENIE

Niniejszym oświadczam, iż mój wkład w publikację:

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OLEJNICZAK, M., DONALDSON, L. W., WĘGRZYN, G., & NEJMAN-FALEŃCZYK, B. (2023).
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PLOS ONE, 18(12), E0296038. [HTTPS://DOI.ORG/10.1371/JOURNAL.PONE.0296038](https://doi.org/10.1371/JOURNAL.PONE.0296038)

polegał na:

kierowaniu jednym z projektów badawczych, w ramach których zostały wykonane badania
opisane w tym artykule, udziale w tworzeniu koncepcji badań, dyskusjach nad interpretacją
uzyskanych wyników doświadczalnych oraz wprowadzeniu poprawek do wstępnej wersji
manuskryptu.



prof. dr hab. Grzegorz Węgrzyn

.....
PODPIS

Bożena Nejman-Faleńczyk

09.04.2026 r.

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IMIĘ I NAZWISKO

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DATA

Katedra Biologii Molekularnej
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AFILIACJA

OŚWIADCZENIE

Niniejszym oświadczam, iż mój wkład w publikację:

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polegał na: (i) współudziale w opracowaniu koncepcji badań nad cząsteczką 24B_1 i określeniu głównych celów badawczych, (ii) współudziale w napisaniu na realizację tych celów projektów badawczych i pozyskaniu funduszy z NCN na przeprowadzenie eksperymentów do niniejszej publikacji, (iii) kierowaniu jednym z projektów i udziale w drugim jako główny wykonawca, (iv) koordynowaniu prac zespołu badawczego, (v) nadzorze nad realizacją poszczególnych etapów prac projektowych oraz podejmowaniu decyzji o zmianach kierunków badań, (vi) współudziale w opracowaniu metod badawczych, (vii) opracowaniu głównych założeń manuskryptu i opisanu uzyskanych wyników oraz poprowadzeniu dyskusji tej pracy.

Jako autor korespondujący odpowiadałam również za kontakt z redakcją czasopisma, proces recenzyjny, wprowadzanie poprawek oraz opracowanie odpowiedzi na uwagi recenzentów. Ponadto czuwałam nad zgodnością pracy z wymogami etycznymi i formalnymi czasopisma oraz nad ostatecznym zatwierdzeniem manuskryptu do publikacji.

B. Nejman-Faleńczyk
.....

PODPIS

Praca nr 3

UpRoi1 - the phage-derived microRNA-size non-coding RNA molecule regulating both phage and its bacterial host development

Bloch, S[#], **Lewandowska, N[#]**, Zwolenkiewicz-Nowak, J., Mazur, M., Chylewska, A., Dąbrowska, A. M., Musiał, N., Czaplewska, P., Wesółowski, W., Łukasiak, A., Ferenc, M., Pietrzycki, M., Węglińska, E., Mach, P., Węgrzyn, G., Olejniczak, M., & Nejman-Faleńczyk, B.

(2026) Biochimica et Biophysica Acta. Gene Regulatory Mechanisms, 1869(1), 195135.

(<https://doi.org/10.1016/j.bbagr.2026.195135>)

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UpRoi1 – the phage-derived microRNA-size non-coding RNA molecule regulating both phage and its bacterial host development

Sylwia Bloch^{a,b,1}, Natalia Lewandowska^{a,1}, Joanna Zwolenkiewicz-Nowak^c, Monika Mazur^c, Agnieszka Chylewska^d, Aleksandra M. Dąbrowska^d, Natalia Musiał^e, Paulina Czaplewska^e, Wojciech Wesołowski^a, Aleksandra Łukasiak^a, Mariusz Ferenc^f, Marcin Pietrzycki^{f,g}, Emilia Węglińska^a, Paulina Mach^a, Grzegorz Węgrzyn^a, Mikołaj Olejniczak^c, Bożena Nejman-Faleńczyk^{a,b,*}

^a Department of Molecular Biology, University of Gdańsk, Wita Stwosza 59, Gdańsk, Poland

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^e Laboratory of Mass Spectrometry-Core Facility Laboratories, Intercollegiate Faculty of Biotechnology UG&MUG, University of Gdańsk, Abrahama 58, Gdańsk, Poland

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Bacterial motility

ABSTRACT

The phage $\Phi 24_B$ -encoded small RNA (sRNA), named UpRoi1, was identified following the prophage induction. The UpRoi1 molecule is encoded in the antirepressor region of the phage genome and is notably small, consisting of only 30 nucleotides. This sRNA is comparable in size to microRNAs found in eukaryotic cells, and thus, unusual for prokaryotic sRNAs. Bioinformatic analysis revealed that UpRoi1 has multiple interaction sites within the phage and host genomes, suggesting its complex regulatory role. In turn, RNA-seq analyses confirmed that the UpRoi1 molecule has a broad impact on the host's gene expression and influences numerous biological processes, particularly those involved in bacterial motility. Furthermore, binding assays revealed that the UpRoi1 molecule may directly interact with the 5' UTR of the phage antirepressor Roi mRNA as well as a bacterial transcript of the *flgL* gene encoding a protein associated with the structural unit of the bacterial flagellum. This affects both phage and host development, facilitating the switch of the phage to the lytic cycle. Besides, the RNA chaperone protein ProQ has been shown to bind the investigated sRNA with its target transcripts. Based on these results, we have found many similarities between UpRoi1 and herpesviral microRNAs, which is of high importance, because it justifies the occurrence of microRNA-type molecules in Shiga toxin-converting phages.

1. Introduction

Shiga toxin-producing *Escherichia coli* strains (STEC) are pathogenic bacteria that produce Shiga toxin as a result of the presence in their genomes Shiga toxin-converting bacteriophages (shortly, Stx phages), carrying *stx* genes. Stx phages (with their most investigated representative, a $\Phi 24_B$ phage) share numerous similarities in their life cycle and genomic organization with bacteriophage λ , which is the most extensively studied member of the lambdoid phages family [1,2]. These

phages are classified as temperate ones, capable of undergoing two developmental pathways, lysogenic and lytic. Shiga toxin production is associated with prophage induction and the subsequent lytic cycle, which can be induced by various factors, such as antibiotics, UV light, or hydrogen peroxide. During the last stage of this cycle, the phage induces lysis of the bacterial host cell, releasing not only progeny virions but also the Shiga toxin into the intestinal lumen [3].

Once inside the human body, the toxin disrupts cellular protein synthesis through ribosome inactivation, leading to cell death and tissue

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damage. STEC can cause severe food poisoning in humans and, in addition, can lead to numerous complications, such as hemorrhagic colitis, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura, which may even result in death [4–6]. Mortality rates reach even 87% among elderly people and children under five years of age, indicating that these groups are particularly vulnerable to severe complications [7]. During the lysogenic phase, most phage genes, including those responsible for Shiga toxin production, are repressed, resulting in the inhibition of toxin synthesis. Consequently, maintaining the phage in the lysogenic cycle (the prophage stadium) offers a potential strategy for mitigating the impact of STEC infections [7].

The regulation of the lysogenic-lytic switch is a complex process, and numerous factors, such as bacterial cell conditions, access to essential nutrients, temperature, and environmental pH, contribute to the maintenance of lysogeny or induction of the phage lytic cycle. However, the determining role in the cycle switch is played by proteins that act as repressors of lytic development (cI, cII, cIII), along with antirepressors (Cro, Ant, Roi, D.ant) that counteract this inhibition [8,9]. The balance between these repressors and antirepressor proteins controls the bacteriophage cycle switch. Inactivating phage repressors in lambdoid phages depends on the host SOS system, which can be triggered by physiological changes in the host cells, like UV light exposure, hydrogen peroxide treatment, or antibiotics. Typically, the inducers of the SOS response lead to DNA damage, activating the RecA protein, which subsequently facilitates the degradation of both the bacterial LexA protein and the phage cI repressor [8].

Other factors that can modulate the levels of antirepressors and repressors in the cell are small, non-coding RNA molecules (sRNA). Previous research on bacteriophage P22 [9] has demonstrated that a small non-coding RNA plays an essential role in regulating phage gene expression. As a result, sRNA may influence the levels of repressors and antirepressors, thereby affecting the choice between alternative phage developmental pathways. Recent studies have shed light on the significant role of small RNAs in prokaryotic systems, where they regulate various physiological processes and stress responses [10,11]. Although prokaryotic sRNAs are less understood compared to their eukaryotic counterparts, their roles in bacteriophage development are becoming increasingly recognized [11–13].

Small non-coding RNA molecules can contribute to the lambdoid phage cycle switch [14]. These sRNAs can function as either *cis*-acting or *trans*-acting regulators, depending on their proximity and complementarity to their target mRNA. *Cis*-acting sRNAs are typically encoded near to their target genes and regulate expression by direct base-pairing with target mRNA, often affecting their stability or translation process. *Trans*-acting sRNAs, on the other hand, are encoded at distinct locations from their target mRNA and usually require additional components, such as RNA chaperones (e.g., Hfq), to form a stable complex [10,11].

Contrary to eukaryotic microRNAs, which are short molecules (20 nucleotides long or so) matured from longer primary transcripts, the majority of prokaryotic sRNAs are longer (a few or several dozen of nucleotides) and transcribed as the mature molecules. However, it appears that biologically active microRNA-like RNAs can occur in bacteria. The first phage-derived microRNA-like molecule (20 nucleotide long), named 24B_1 and produced due to processing of a longer (80 nucleotide) primary transcript, has been discovered and demonstrated to influence the expression of phage genes [14]. Recent findings demonstrated that its processing involves the action of the Hfq protein, which binds to the precursor but not the mature form [15]. Moreover, apart from controlling phage development directed toward the lysogenic cycle, 24B_1 can also modulate host gene expression and influence bacterial metabolism, affecting key bacterial functions. The presence of this molecule is crucial for reprogramming host energy metabolism and maintaining the prophage state. Interestingly, this phage sRNA was presented as the first microRNA-type molecule of phage origin, which shares similarities with herpesviral microRNAs [14,15].

Our search for other small RNA molecules of phage origin led to the

discovery of another sRNA, named UpRoi1. This sRNA molecule is only 30 nucleotides long and has been characterized in detail in this work. In addition to the physiological effects of this molecule overproduction, we also show its direct interaction with phage and bacterial transcripts and its dependence on RNA chaperone, the ProQ protein.

Finally, based on the obtained results, we consider UpRoi1 sRNA in terms of its similarity to herpesvirus microRNA molecules. The idea of searching for similarities with viral microRNAs arose earlier, during research on the 24B_1 molecule - the first discovered phage non-coding RNA of microRNA-size [14,15]. In this work, we take a closer look at the newly identified UpRoi1 molecule, and also widely study it in this context in the Discussion section.

2. Results

2.1. Identification of a small, microRNA-like UpRoi1 molecule of the phage origin

Following induction of the Φ 24_B prophage in the *E. coli* host with 1 μ g/ml mitomycin C, transcriptomic analyses revealed the presence of a small, 30-nt long RNA (Fig. 1A, B). This molecule is encoded by the *uproi1* gene, located in the regulatory region of the phage genome, between *ant* and *roi* genes, but in the opposite orientation to those latter ones (Fig. 1C). Relevant promoters and terminators could also be detected. Quantitation of the microRNA-type molecule, named UpRoi1, was assessed by RT-qPCR after prophage induction (both spontaneous and forced by the addition of mitomycin C) in host bacterial strain MC1061 bearing pBAD24, pBAD24_UpRoi1, and pBAD24_pN_UpRoi1. The *E. coli* (Φ 24_B) [pBAD24_pN_UpRoi1] variant differs from the *E. coli* (Φ 24_B) [pBAD24_UpRoi1] one by the presence of a natural pN promoter in the plasmid. Moreover, only in the former variant does overproduction of UpRoi1 occur naturally, as there is no external forcing of the *uproi1* expression from the plasmid (no promoter inducer is needed). In turn, the *E. coli* (Φ 24_B) [pBAD24_UpRoi1] variant carries the *uproi1* gene under pBAD promoter, but this promoter remained inactive in all experiments as no inducer (arabinose in this case) was added. To overcome the common problem of promoter leakage, we tested two systems and chose the pBAD system with an arabinose promoter that turned out to be less leaky than the pUC system with the lac promoter. In addition to the control variant with the empty vector pBAD24, the strain carrying plasmid pBAD24_UpRoi1 was used in most of the experiments as an additional control, constituting a background for the activity of the natural pN promoter.

The quantitation analysis revealed that, after 60 min from the addition of mitomycin C, levels of the investigated small RNA were increased in all variants (relative to time 0), with the highest level in the pBAD24_pN_UpRoi1 variant, where expression of the molecule occurs from its natural pN promoter (Fig. 1D). The significantly lower but still detectable UpRoi1 transcript level in the remaining variants *E. coli* (Φ 24_B) [pBAD24], and *E. coli* (Φ 24_B) [pBAD24_UpRoi1], seems to be a result of *uproi1* gene expression from the prophage. As can be seen, the UpRoi1 level is lower under spontaneous induction conditions when prophage excision occurs spontaneously and mitomycin C is not used. Importantly, under such conditions, the level of *uproi1* gene expression was still significantly higher in the UpRoi1-overproducing variant than in the other two variants, which may indicate its low but constitutive expression from the vector carrying pN promoter. These results correlate with the calculated spontaneous induction frequency estimated from PFU release, which was equal to 6.05×10^{-5} of induction events per cell per generation for the variant with UpRoi1 overproduction and higher in comparison with 1.83×10^{-6} and 1.21×10^{-6} events per cell per generation for the *E. coli* (Φ 24_B) [pBAD24] and *E. coli* (Φ 24_B) [pBAD24_UpRoi1] variants, respectively. These results confirm the production of active UpRoi1 from the vector despite the absence of the inducer, mitomycin C. In contrast, after inducer addition, *uproi1* gene expression efficiency increased in all tested variants, but was still the highest in the

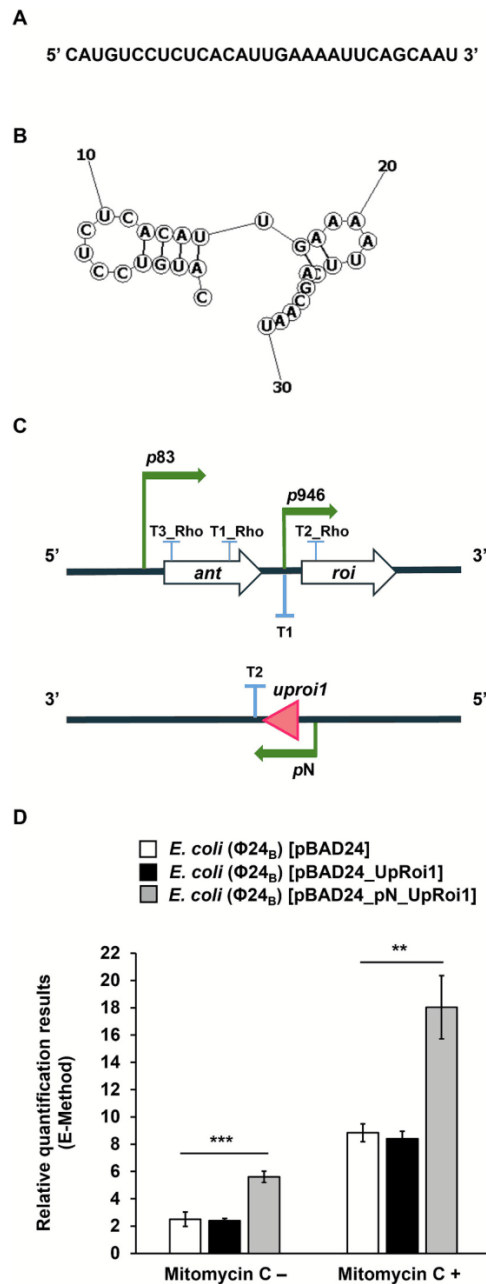


Fig. 1. Identification and expression of phage UpRoi1 sRNA. Sequence (A) and secondary structure of UpRoi1 generated by the RNAstructure program (B). The sequence of UpRoi1 sRNA was identified in the results of NGS analysis of the fraction of small RNAs less than 50 nucleotides in length that were isolated from *E. coli* lysogens after induction of Φ24_B prophage. (C) Localization of the *uproi1* gene (red triangle) in the genome of *E. coli* (Φ24_B) lysogen. UpRoi1 is antisense to 5' UTR of *roi* gene and its gene is encoded on the complementary DNA strand to the *roi* sequence (*cis*-encoded sRNA). Black arrows indicate the position of *ant* and *roi* genes coding for antirepressors, whose action favors the lytic cycle. The identified promoters and terminators of indicated genes are marked by *p* and *T*, respectively, with appropriate numbers. Sequences of the *pN* promoter -35 and -10 boxes are TTGTGTA and GTTTATTTT, respectively, and were identified by BPROM software. Analysis with BPROM also allowed for predicting TF binding sites within the *pN* promoter sequence for TyrR (AATATATA), LexA (TATATACA and ATATATAC), RpoD14 (TTGTGAAT), SoxS (ATTGTGTA), and ArgR2 (TTTATTTT) transcriptional regulators. The T1 and T2 Rho-independent terminators have been identified by ARNold software, and their sequences are presented in Supplementary Fig. S2. In turn, Rho-dependent terminators T1_Rho, T2_Rho, and T3_Rho were predicted by the RhoTermPredict program, and its sequence is presented in Supplementary Table S3 (D). Detection and quantitation of UpRoi1 microRNA-tape molecule assessed by RT-qPCR after prophage induction (both spontaneous and forced with 1 μg/ml mitomycin C) in host bacteria bearing pBAD24, pBAD24_UpRoi1, or pBAD24_pN_UpRoi1 vectors. Levels of the UpRoi1 transcript were determined at 60 min after prophage induction with mitomycin C (+) and in variants presenting spontaneous induction level (-) and normalized to time zero when the culture reached the Od value = 0.1. The presented results are mean values from three independent experiments with error bars indicating SD.

E. coli ($\Phi 24_B$) [pBAD24_pN_UpRoi1] strain.”

Importantly, there was no detectable production of the UpRoi1 in the absence of a prophage in non-lysogen strains *E. coli* [pBAD24], *E. coli* [pBAD24_UpRoi1], and *E. coli* [pBAD24_pN_UpRoi1] (Supplementary Fig. S1). Apparently, some phage factors are required for efficient production of the mature UpRoi1 molecule, therefore, we decided not to analyze further non-lysogen variants in this work.

2.2. Identification of target binding sites for UpRoi1 sRNA

To identify potential binding sites for the UpRoi1 molecule, we employed a novel bioinformatics approach based on the original algorithm presented for the first time in this study (Supplementary Table S1). The methodology we developed derives from the nature of the phage-host interaction phenotype problem. More specifically, having modeled a functional vector space that reconstructs the phage-host interaction, we were able to propose several hypotheses as derivatives

of this model. These hypotheses encompassed both individual interactions and complex molecular pathways. In this particular case, we concentrated on identifying potential target sites for UpRoi1 in the genome of *E. coli* bacteria carrying the Stx phage. We obtained the top 30 sequences of 30-nt length each (Supplementary Table S1). Among the acquired data, we selected 12 sites located within 9 bacterial and 3 phage genes whose mRNA may potentially form a complex with the tested sRNA, as indicated by IntaRNA software. Notably, the UpRoi1 binding site was found in the untranslated regions in four cases. All of these target binding sites were gathered in Supplementary Table S2, and finally, three of them, located within 5' UTR *roi*, *figL*, and *oppA* genes and showing high hybridization energy, were analyzed in detail below.

2.3. Effects of UpRoi1 on bacteriophage development

To study the effects of UpRoi1 on bacteriophage development, plasmids carrying the additional copy of the *uproi1* gene (either with or

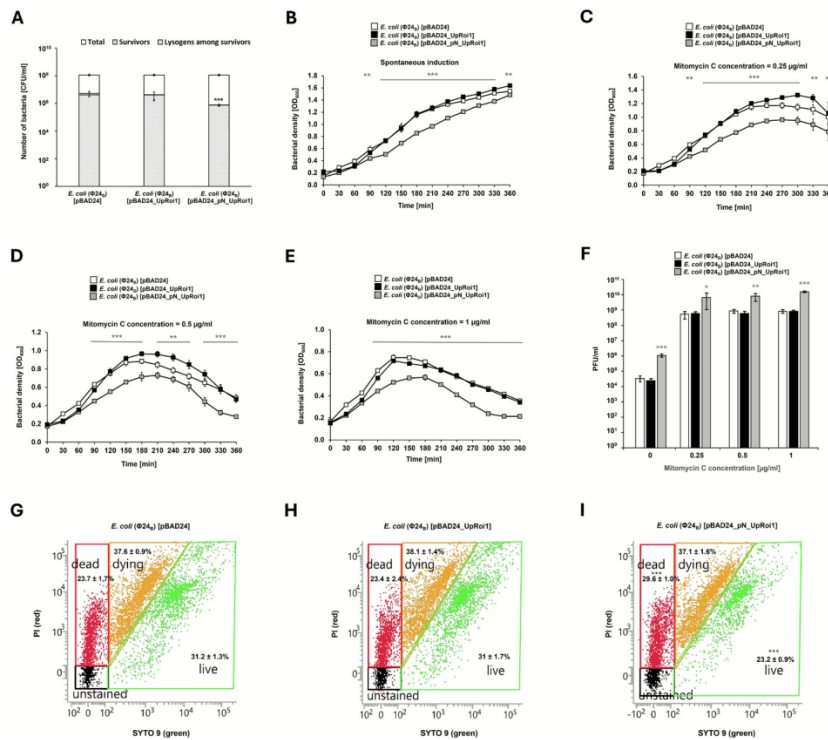


Fig. 2. Effects of UpRoi1 sRNA on bacteriophage development. (A) The survival of *E. coli* [pBAD24], *E. coli* [pBAD24_UpRoi1], and *E. coli* [pBAD24_pN_UpRoi1] bacteria and their lysogenization efficiency after infection with phage $\Phi 24_B$. The bars represent the number of bacterial cells in the control variant without the addition of bacteriophage $\Phi 24_B$ (white), the number of cells that survived infection with phage $\Phi 24_B$ (light gray), and the number of lysogens (checked pattern). The experiment was performed at $m.o.i = 5$. Statistically significant differences between survivors or lysogens obtained in *E. coli* [pBAD24_pN_UpRoi1] and those from two other variants without natural pN promoter (*E. coli* [pBAD24], *E. coli* [pBAD24_UpRoi1]) were determined by the Student's *t*-test and marked with the symbol of three asterisks (***) for $p < 0.001$. (B,C,D,E) The changes in OD values of bacterial cultures *E. coli* ($\Phi 24_B$) [pBAD24], *E. coli* ($\Phi 24_B$) [pBAD24_UpRoi1], and *E. coli* ($\Phi 24_B$) [pBAD24_pN_UpRoi1] measured at the indicated times after (B) spontaneous induction of prophage or (C,D,E) induction forced by mitomycin C (added in concentration 0.25, 0.5 or 1 µg/ml, respectively). At panels C, D, E, mitomycin C was added at time zero after the sample was withdrawn. Statistically significant differences between analyzed variants were determined by the Student's *t*-test and marked with the symbols of asterisks (***) for $p < 0.001$, (**) for $p < 0.01$, and (*) for $p < 0.05$. Gray stars indicate statistically significant differences between the variant producing UpRoi1 molecule (*E. coli* ($\Phi 24_B$) [pBAD24_pN_UpRoi1]) and both control variants *E. coli* ($\Phi 24_B$) [pBAD24] and *E. coli* ($\Phi 24_B$) [pBAD24_UpRoi1]. (F) The number of phage particles (PFU) per ml of culture, estimated at 360 min after prophage induction. Statistically significant differences between *E. coli* [pBAD24_pN_UpRoi1] and two other variants without natural pN promoter (*E. coli* [pBAD24], *E. coli* [pBAD24_UpRoi1]) were determined by the Student's *t*-test and marked with the symbols of asterisks (***) for $p < 0.001$, (**) for $p < 0.01$, and (*) for $p < 0.05$. (G, H, I) Flow cytometry analysis of bacterial cells *E. coli* ($\Phi 24_B$) [pBAD24] (G), *E. coli* ($\Phi 24_B$) [pBAD24_UpRoi1] (H), and *E. coli* ($\Phi 24_B$) [pBAD24_pN_UpRoi1] (I) performed at 300 min after prophage induction with mitomycin C (1 µg/ml). Bacteria were stained with a LIVE/DEAD BacLight Bacterial Viability Kit. PI (red) fluorescence is plotted against SYTO 9 (green) fluorescence. Representative dot-plots are presented and percentage values averaged from 3 independent repetitions are given for dead (red), dying (orange), and live (green) bacteria in each tested variant. Statistically significant differences between live or dead fractions of *E. coli* [pBAD24_pN_UpRoi1] cells and the other two variants: *E. coli* [pBAD24] and *E. coli* [pBAD24_UpRoi1], were determined by the Student's *t*-test and marked with the symbol of three asterisks (***) for $p < 0.001$. The presented results are mean values from three independent experiments with error bars indicating SD.

without its natural *pN* promoter) were applied. The rationale for testing overexpression rather than deletion of the *upr01* gene was based on the fact that *upr01* partially overlaps the *roi* gene, located at the opposite strand and encoding a potential target of UpRoi1. Therefore, destroying the putative target would diminish the probability of identifying the biological functions of the tested molecule.

When testing the number of bacteria surviving phage infection, almost all surviving bacteria in the *E. coli* ($\Phi 24_B$) [pBAD24_UpRoi1] and *E. coli* ($\Phi 24_B$) [pBAD24_pN_UpRoi1] variants turned out to carry the $\Phi 24_B$ prophage. Importantly, in the UpRoi1-overproducing variant, the fraction of surviving bacteria was also significantly lower (0.7 orders of magnitude) compared to the other two variants (Fig. 2A). In turn, when monitoring the density of the bacterial culture after prophage induction (spontaneous – Fig. 2B or forced by mitomycin C added in three different concentrations: 0.25, 0.5 and 1 $\mu\text{g}/\text{ml}$ – Fig. 2C, D, E, respectively) we found that overproduction of UpRoi1 from its own promoter *pN* (*E. coli* ($\Phi 24_B$) [pBAD24_pN_UpRoi1]) causes significantly more effective lysis of bacteria, relative to the control strains *E. coli* ($\Phi 24_B$) [pBAD24] and *E. coli* ($\Phi 24_B$) [pBAD24_UpRoi1]. 360 min after the induction with 1 $\mu\text{g}/\text{ml}$ of mitomycin C, the OD value was twice lower in the overproducing variant than in the control variants (Fig. 2E). Moreover, as indicated in Fig. 2F, the number of phage particles (PFU/ml) in the UpRoi1-overexpressing variant after prophage induction with tested concentrations of mitomycin C also increased significantly (~10-fold) at the end of the experiment (360 min) as compared to the control variants *E. coli* ($\Phi 24_B$) [pBAD24] and *E. coli* ($\Phi 24_B$) [pBAD24_UpRoi1]. Besides, the more efficient killing of host bacteria *E. coli* ($\Phi 24_B$) [pBAD24_pN_UpRoi1] bearing the *upr01* overexpressing plasmid was corroborated

by results of the flow cytometry experiments, indicating that overproduction of UpRoi1 resulted in a decreased fraction of living cells, up to 23% as compared to bacteria lacking an active *upr01* gene on plasmid, which remained at the level of 31% (Fig. 2G–I).

The results presented in this section indicate that UpRoi1 promotes phage lytic development and more effectively kills the host bacteria after prophage induction.

2.4. Molecular mechanism of regulation of bacteriophage development by UpRoi1 based on its interaction with the 5'UTR of the *roi* gene

To learn about the molecular mechanism of UpRoi1-mediated regulation of bacteriophage development, we tested potential molecular targets of this microRNA-like molecule. Phage $\Phi 24_B$ genome sequence analysis and *upr01* gene localization suggested that UpRoi1 sRNA may act as a *cis*-regulatory element and hybridize with full complementarity with the 5'UTR region of the *roi* gene transcript, encompassing the *p946* promoter and T1 Rho-independent terminator identified by ARNold software (see Fig. 1C and Supplementary Table S2, position 9). Importantly, the UpRoi1 binding site is located between two other Rho-dependent terminators T1_Rho and T2_Rho (predicted by RhoTermPredict program) which may inhibit the transcription from *p83* and *p946* promoters, respectively. (Fig. 1C and Supplementary Fig. S2). Therefore, we examined interactions between UpRoi1 and 100-nt long RNA fragment encompassing the region between *ant* and *roi* coding sequences, which localization, sequence, and secondary structure are presented in Supplementary Fig. S2. Besides, since interactions between bacterial RNA molecules are often modulated by RNA chaperone

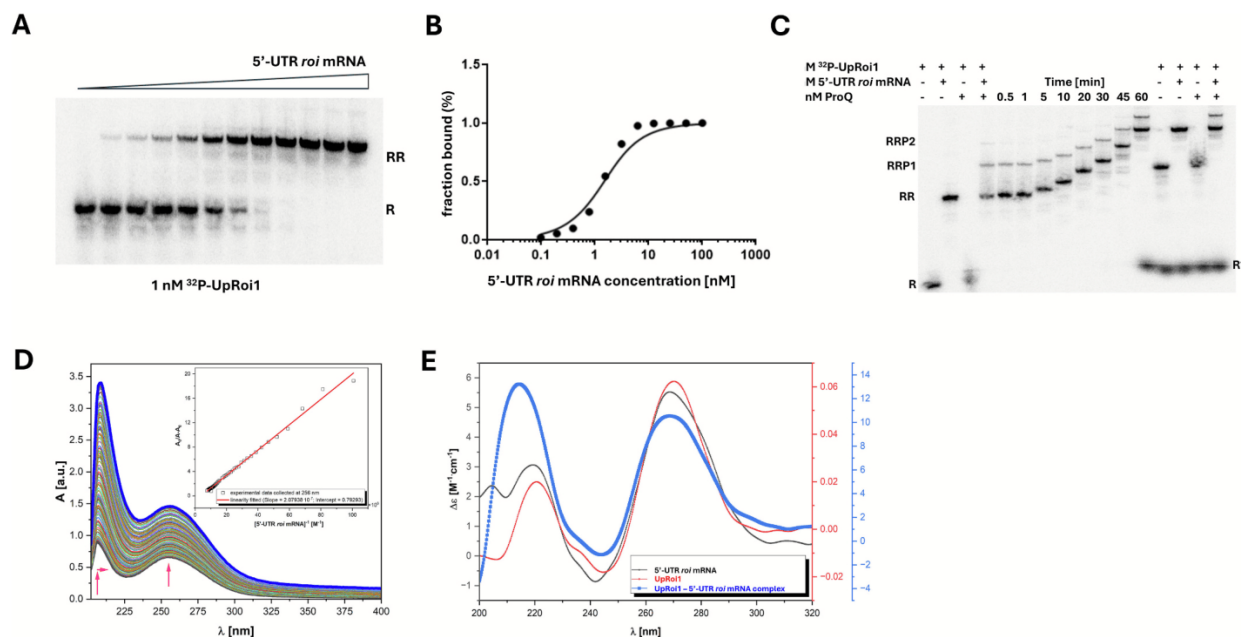


Fig. 3. The direct interaction between UpRoi1, 5'-UTR *roi* mRNA, and ProQ protein, assessed by molecular and spectroscopic methods. (A) The pairing of the UpRoi1 sRNA to the 5'UTR *roi* mRNA, monitored using a gelshift assay. Unbound ^{32}P -UpRoi1 sRNA is marked as R, and sRNA-mRNA complex as RR. (B) The fraction-bound data from panel A, plotted versus RNA concentration. (C) The association of ^{32}P -labeled UpRoi1 RNA to 100-nt long fragment of the 5'-UTR of *roi* mRNA in the presence of the ProQ protein. The binding was monitored using a gelshift assay. The control reactions are located on the left and right sides of the gel, except that the time of incubation was the same as the longest time point of the time course, and they were loaded immediately after the last time point, to provide migration markers. The unbound fragment of ^{32}P -UpRoi1 is marked as R, the complex of the 5'-UTR of *roi* mRNA with ^{32}P -UpRoi1 as RR, the complex of the 5'-UTR of *roi* mRNA with ^{32}P -UpRoi1 and ProQ protein as RRP. In addition, in the last five lines of the gel (at the bottom), there are visible degraded fragments of the labeled UpRoi1 (marked as R*) which have not exited the gel yet. (D) Absorption spectra of UpRoi1 (0.73 μM ; 0.5 ml) in the presence of increasing amounts of 5'-UTR *roi* mRNA solution (0.031 μM). The direction of the arrow indicates the hyperchromic effect resulting from intermolecular interactions. Inset: plot of $A_{250}/(A_{250} - A_{260}) = f(1/[5'\text{-UTR } roi \text{ mRNA}])$ established as a result of interactions studied in Tris buffer (pH 7.5). (E) Circular dichroism (CD) spectra of the adequate RNA molecules UpRoi1 in red (0.73 μM), 5'-UTR *roi* mRNA in black (0.031 μM), and their complex in blue (0.132 μM). All assays were performed in three independent replicates.

proteins, we have tested the effects of the ProQ and Hfq proteins on this interaction. The obtained data showed that the 100-nt long 5'-UTR *roi* mRNA fragment bound to UpRoi1 molecule as indicated by the band shifts (RR complex) in Fig. 3A, and the bound fraction increased with the increasing concentration of the mRNA molecule (Fig. 3B). The data also indicated that ProQ bound to the complex of 5'-UTR fragment of *roi* mRNA and UpRoi1 sRNA (RRP complexes in Fig. 3C). Moreover, the presence of a small amount of the second RRP may indicate the formation of a more complex but unstable structure (Fig. 3C). This assay also showed how individual complexes migrated when applied to the gel at different time points (0.5, 1, 5, 10, 20, 30, 45, and 60 min). Samples added after 60 min (the last five lanes) were applied as the latest, hence they are located at the very top of the gel. Importantly, in these lanes, at the bottom of the gel, there are visible degraded fragments of the labeled UpRoi1 (R^*) which have not exited the gel yet. These fragments are not visible in samples applied at earlier time points, as they have migrated in the gel for a longer time (Fig. 3C). Curiously, we didn't observe interaction between the Hfq protein and the tested RNA molecules (data not shown).

To confirm these observations, the interaction of UpRoi1 and 100-nt long 5'-UTR *roi* mRNA was also tested by spectroscopic methods (Fig. 3D and E). UV electronic spectra showed distinct changes upon interaction with UpRoi1 and 5'-UTR *roi* mRNA molecules (bathochromic shift of approximately 205 nm and hyperchromic effect at 260 nm). The absorbance values (A) increased with increasing concentrations of the 5'-UTR *roi* mRNA component, suggesting molecular interactions (Fig. 3D). This was particularly evident near the absorption maximum, where the gradual increase in the formation of a complex of the RNA strands was observed. The binding constant (K_{bind}) for the interaction between UpRoi1 and 5'-UTR *roi* mRNA was determined at the level of

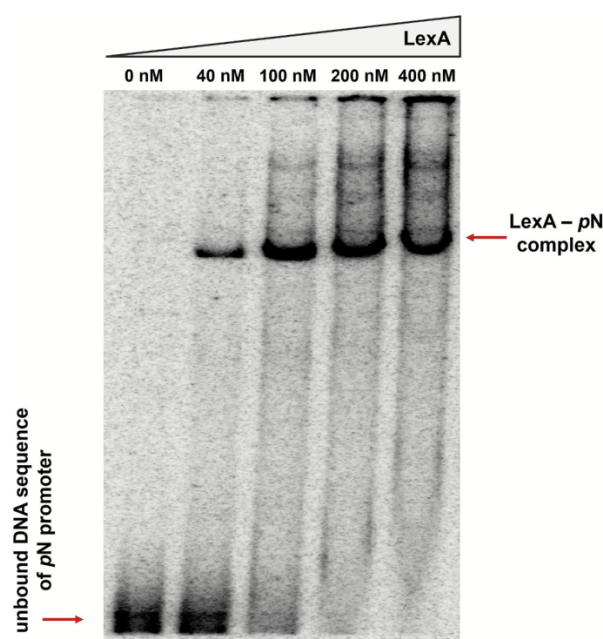


Fig. 4. The binding of the transcription factor, the LexA protein, to the pN promoter sequence of the *uproil* gene revealed by the gelshift assay. The complex of LexA protein and pN promoter as well as the unbound pN promoter DNA sequence, are indicated by arrows. The LexA protein was used in the increased concentrations of 40, 100, 200, and 400 nM. Two binding sites for LexA protein (TATATACA and ATATATAC) were predicted bioinformatically by BPROM software near the -35 box of pN promoter sequence as indicated in Supplementary Fig. S2. This experiment was performed in three independent replicates.

$3.81 \cdot 10^6 \text{ M}^{-1}$ and this value reflects moderate-to-strong binding affinity with a good linearity correlation parameter ($R^2 = 0.99459$; 298 K). The additionally designated value of $\Delta G = -37.52 \text{ kJ/mol}$ confirmed that the interaction was spontaneous, thermodynamically favorable, and stable in the tested conditions (Fig. 3D).

In turn, CD spectral analysis provided additional information that the length and interactions between RNA fragments determine their structure and degree of ordering. Short RNA UpRoi1 remains relatively unstable, whereas a 100-nt long 5'-UTR *roi* mRNA fragment exhibits clear helical features (Fig. 3E). In more detail, CD spectrum analysis of the short UpRoi1 RNA (red line in Fig. 3E) revealed a weak characteristic maximum around 270 nm ($\Delta\epsilon$ 0.0622), along with a shallow minimum ($\Delta\epsilon$ -0.018) with low ellipticity in the 240–245 nm range. This profile suggests that the 30-nucleotide RNA is unlikely to form a stable A-type helix, which is characteristic of well-ordered RNA structures. Instead, it resembles unstable, randomly coiled fragments or short segments with limited spatial organization, such as simple hairpins or loops. This observation may explain the presence of degraded fragments of the UpRoi1 molecule in Fig. 3C, the length of which is insufficient for the creation of a complex stable secondary structure. In contrast, the CD spectrum of the longer, 100-nt long 5'-UTR *roi* mRNA fragment shows no particular departures from the classic spectrum of the A-form helical RNA. The distinct maximum ($\Delta\epsilon$ 5.523) near 270 nm and the deep minimum ($\Delta\epsilon$ -0.863) around 242 nm, together with a significantly higher signal amplitude, indicate the presence of stable, well-formed secondary structures (black line in Fig. 3E). This suggests that this fragment contains extensive base-paired regions that form helices as well as other structural elements that confer an ordered conformation. However, the most crucial result, confirming our observations from the gelshift binding assay, is the spectrum of the complex formed by combining UpRoi1 and the 5'-UTR *roi* mRNA fragment. The occurrence in the CD spectrum of the strongest signals, such as deeper minima ($\Delta\epsilon$ -1.141) and higher maxima ($\Delta\epsilon$ 10.539), compared to the individual RNA molecules – confirmed the sRNA–mRNA complex formation (blue line in Fig. 3E) with significant structural order. It is likely due to the formation of extended A-form helices, which are stable as they involve more base pairing, thereby enhancing the conformational and functional stability of RNA molecules.

When analysing the promoter-terminator region of the *ant-roi* fragment of the phage $\Phi 24_B$ genome, we detected putative binding sites TATATACA and ATATATAC of the LexA protein near the -35 box of the pN promoter (Supplementary Fig. S2). Since LexA is a repressor of the SOS regulon, whose activation is also important for the prophage induction, we have tested the binding of LexA to the pN promoter region. As indicated by the gelshift assay, LexA can bind to the tested promoter relatively strongly, indicating that it may be an important factor for the regulation of the *uproil* gene expression (Fig. 4).

The results described in this section suggest a molecular mechanism of the UpRoi1-mediated regulation of bacteriophage $\Phi 24_B$ development, especially the promotion of lytic development over the maintenance of lysogeny. The proposed mechanism is described in more detail in the Discussion. It can be based on the direct regulation of the *roi* gene, as when analysing RNA-seq results, we captured a relatively small ($\log_2$ FoldChange = 0.28) but meaningful up-regulation of the *roi* gene expression in the UpRoi1-overproducing variant *E. coli* ($\Phi 24_B$) [pBAD24.pN.UpRoi1] in comparison to control *E. coli* ($\Phi 24_B$) [pBAD24]. Importantly, the expression of genes coding for the other two antirepressors, Ant and D_{ant}, was also slightly increased in the former variant (Supplementary Table S4). To confirm these results, the RT-qPCR method was applied, and revealed the higher expression of all antirepressor genes (*cro*, *roi*, *ant*, and *d_{ant}*) in *E. coli* ($\Phi 24_B$) [pBAD24.pN.UpRoi1] relative to the control variant (Fig. 5D -described in detail below).

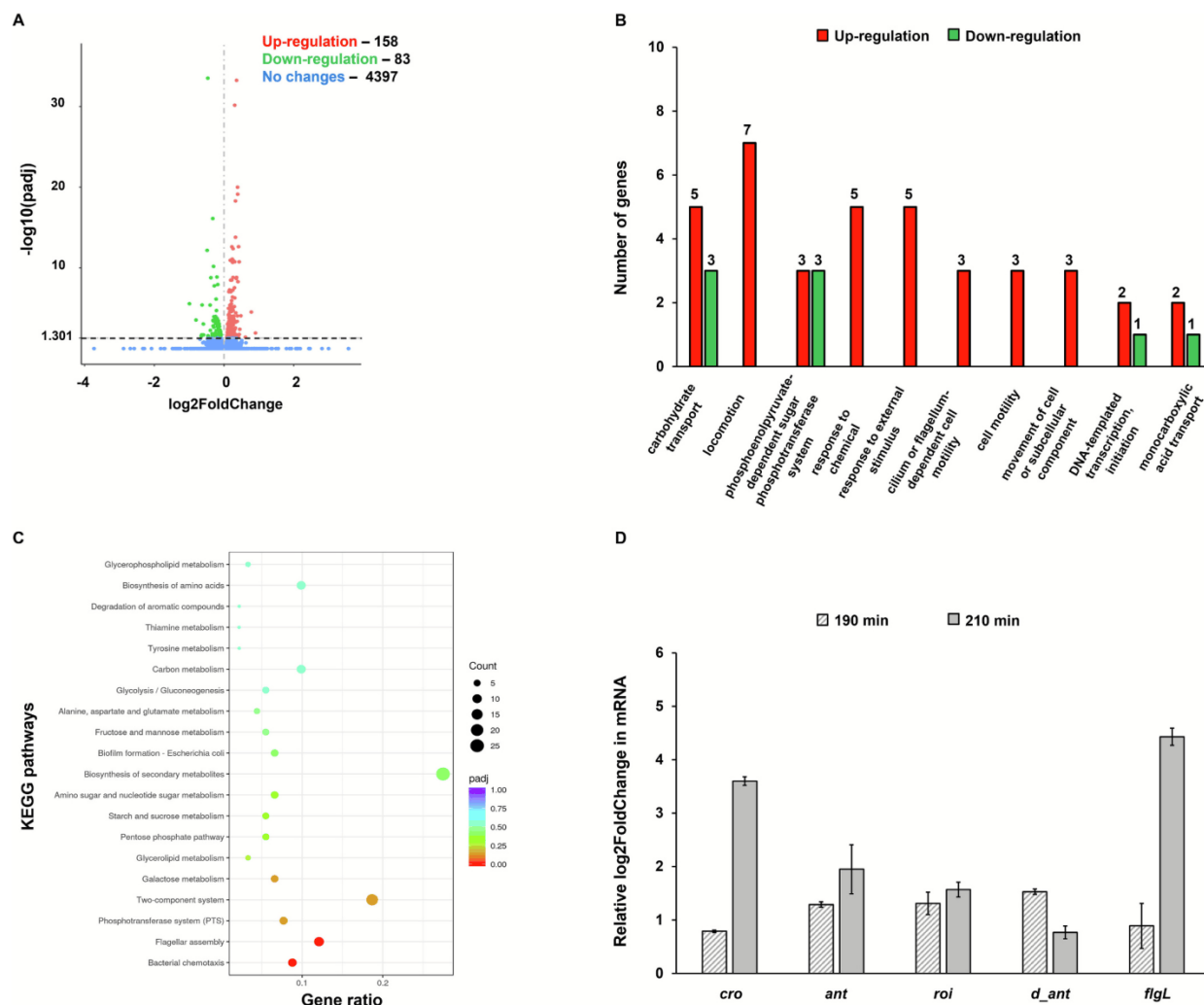


Fig. 5. Analyses of differentially expressed genes in the *E. coli* ($\Phi 24_B$) [pBAD24_pN_UpRoi1] vs. control *E. coli* ($\Phi 24_B$) [pBAD24]. Results were generated by RNA-seq from samples withdrawn at 210 min after mitomycin C-mediated induction of the phage lytic cycle. (A) Volcano plot representation. The x-axis represents $\log_2$ FoldChange, while the y-axis represents the statistical significance for each gene, expressed as $-\log_{10}$ adjusted p -values ($p_{adj} < 0.05$). The colored dots represent the most significant up-regulated (red) and down-regulated (green) genes indicated in Supplementary Table S4. Blue dots indicate genes with no changes in expression, and a horizontal dotted black line indicates the fold-change cut-off (1.301). (B) Gene ontology analysis of differentially expressed genes. The x-axis indicates the pathways' names. The y-axis shows the number of up-regulated (red) and down-regulated (green) genes in *E. coli* ($\Phi 24_B$) [pBAD24_pN_UpRoi1] variant in comparison to the control sample *E. coli* ($\Phi 24_B$) [pBAD24]. (C) KEGG pathway enrichment analysis for all differentially expressed genes. The x-axis indicates the Gene ratio, and the degree of KEGG pathway enrichment. The y-axis indicates the names of the KEGG pathways. The dot size reflects the gene number, whereas the dot color indicates the p -value. The top 20 KEGG pathways are shown. (D) Changes in levels of selected phage and bacterial transcripts in *E. coli* MC1061 ($\Phi 24_B$) [pBAD24_pN_UpRoi1], assessed by RT-qPCR method, relative to control variant *E. coli* MC1061 ($\Phi 24_B$) [pBAD24] at times 190 and 210 min after prophage induction with mitomycin C. All assays were performed in three independent replicates.

2.5. Effects of UpRoi1 on bacterial transcriptome and proteome

As described above, we have demonstrated that UpRoi1 interacts with bacteriophage-encoded RNA, modulating phage gene expression and development. These regulatory events were based on *cis*-mediated interaction with transcript originating from the same locus but transcribed in the opposite direction. However, we also investigated whether UpRoi1 might influence bacterial host gene expression by acting in *trans*.

UpRoi1 was overproduced from a plasmid bearing the *uproi1* gene, and the RNA-seq transcriptome was compared to that obtained from control cells (not overexpressing this gene). Additionally, the $\Phi 24_B$ prophage was induced with mitomycin C to assess conditions of the

switch from lysogenic to lytic mode of phage development. When comparing cells overproducing UpRoi1 (*E. coli* ($\Phi 24_B$) [pBAD24_pN_UpRoi1]) and those which not produce it (*E. coli* ($\Phi 24_B$) [pBAD24]), we distinguished 158 up-regulated and 83 down-regulated genes in cells bearing the *uproi1*-overexpressing plasmid, as shown in the volcano plot (Fig. 5A) and listed in Supplementary Table S4. When looking at the genes with expression changed in response to UpRoi1 overproduction, those related to specific processes could be distinguished, including locomotion (GO:0040011), in which the expression of 7 functional bacterial genes (*cheA*, *cheW*, *tar*, *tap*, *tsr* coding for chemotaxis proteins, and *fliC* and *fliG* encoding flagellar proteins) was increased compared to the control (Fig. 5B). When KEGG pathway enrichment analysis for up-regulated genes in the *E. coli* overproducing UpRoi1 was conducted,

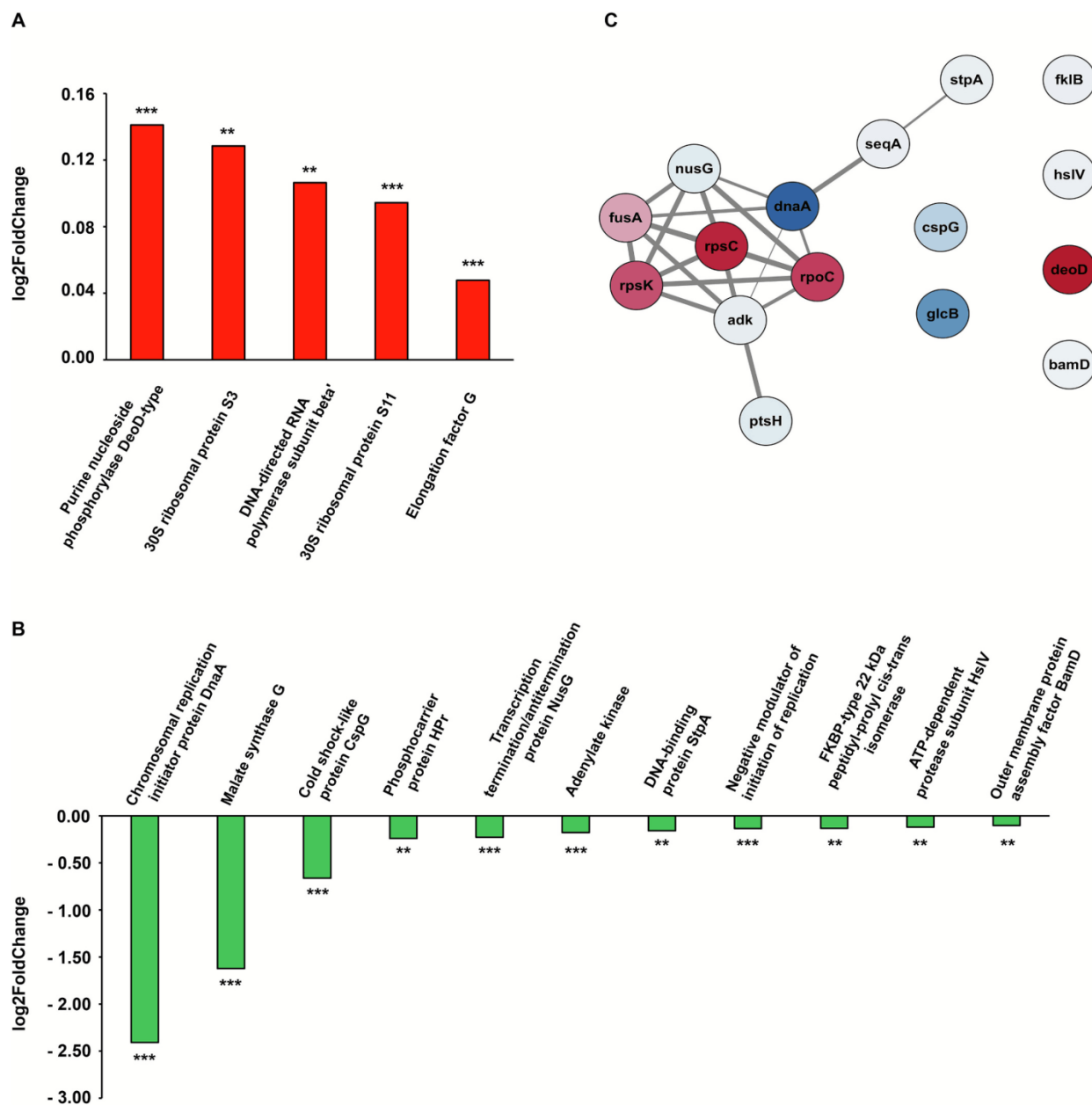


Fig. 6. Results of the proteomic analysis. Proteins with significantly increased (A) and decreased (B) levels (at least $p < 0.01$) in *E. coli* ($\Phi 24_B$) [pBAD24_pN_UpRoi1] relative to control sample *E. coli* ($\Phi 24_B$) [pBAD24] induced with mitomycin C (1 $\mu\text{g}/\text{ml}$). The LC-MS/MS data set was generated from samples withdrawn at 210 min after induction of the phage lytic cycle. The x-axis indicates the proteins' names, whereas the y-axis represents log2FoldChange separately for increased (red columns) and decreased (green columns) protein levels. (C) String network interaction analysis. Upregulated proteins are marked in red, and down-regulated proteins are marked in green. The intensity of the fill correlates with the fold change values - the darker the color, the higher the FC value. The presented results are from three independent experiments.

the *flagellar assembly* (eco02040) and *bacterial chemotaxis* (eco02030) were among the most significantly affected processes. There were 8 genes (*cheA*, *motA*, *tar*, *motB*, *tap*, *tsr*, *cheW*, *cheR*) up-regulated in the *bacterial chemotaxis* pathway and 10 functional genes (*fliC*, *motA*, *motB*, *fliD*, *flgL*, *flgK*, *fliA*, *fliS*, *flgN*, *flgM*) in the *flagellar assembly* pathway. Interestingly, no down-regulated genes were found in these pathways (Fig. 5C). In addition to this, the most important genes for this analysis, like those encoding phage repressor *cl*, phage antirepressors: *Cro*, *Ant*, *Roi*, *D_ant*, and bacterial flagellar protein *FlgL* were tested by the RT-

qPCR technique, which confirmed their upregulation in *E. coli* variant overproducing UpRoi1 (Fig. 5D). The comparison of results assessed by RNA-seq and RT-qPCR techniques is presented in Supplementary Table S5 and indicates the same direction of changes, but reveals different values, which may result from specific technical aspects of both methods. Interestingly, the RT-qPCR results indicated that among the antirepressor genes, the greatest change in expression efficiency was observed for the *cro* gene ($\text{Log}_2\text{FoldChange} = 3.6 \pm 0.08$), whose product was also reported to counteract the *cl* repressor in the lambda

phage [3]. Our assumptions were also confirmed by the unchanged level of *ci* transcript (noticed in both RNA-seq and RT-qPCR assays), indicating that the regulation did not occur at the mRNA level.

When proteomic analyses were performed using the same experimental system, some proteins were found to be especially highly abundant in cells overexpressing UpRoi1. These proteins included those related to nucleic acids metabolism and ribosomal activity (Fig. 6A). On the other hand, the most significantly down-regulated protein in UpRoi1-rich cells was the DnaA protein, the initiator of chromosomal DNA replication (Fig. 6B). The relevance of such a regulation can be attributed to the need to suppress host chromosome DNA synthesis after prophage induction and subsequent intensive replication of the viral genome. Among that, the data were analyzed using a String server, and the visualization of the results in the form of an interaction network is presented in Fig. 6C. The presented graph accounts for up- and down-regulated proteins and their interactions. The most upregulated and interacting proteins turned out to be those linked to transcription and translation processes, such as small ribosomal subunit proteins RpsK and RpsC, and RNA polymerase subunit RpoC.

2.6. Direct interaction of UpRoi1 and bacterial *flgL* mRNA, and phenotypic effect of this regulation in *E. coli* cells

Analysis of the transcriptome and proteome of *E. coli* bacteria allowed us to determine cellular processes and genes potentially regulated by UpRoi1. However, based on these results, we were unable to identify genes directly regulated by this molecule, as the observed effects could also arise from the indirect action of the tested sRNA. To overcome this problem, we compared the obtained results with the data

of bioinformatic searches (see the Section 2.2 and Supplementary Tables S1 and S2). This analysis allowed us to narrow down the list of genes potentially regulated by UpRoi1 and identify those that may be regulated directly (Supplementary Table S2). The compatibility of transcriptomic and bioinformatic data allowed us to select the bacterial *flgL* gene for further analysis. This gene encodes a flagellar hook-filament junction protein and belongs to the *flagellar assembly* pathway (eco02040), whose genes (including *flgL*) are up-regulated in the variant with UpRoi1 overproduction, as indicated by the RNA-seq results (Supplementary Table S4) and RT-qPCR method (Fig. 5D). Besides, as predicted by IntaRNA, there is a direct binding site for UpRoi1 within *flgL* mRNA, and these molecules form a very stable complex with a hybridization energy equal to -23.9 kcal/mol. In addition, IntaRNA visualization of the UpRoi1-*flgL* mRNA allowed us to identify a seed region typical of eukaryotic microRNAs, localized between 2 and 8 nucleotides at the 5' end of sRNA (for details see Supplementary Table S2 and Supplementary Fig. S3). Additionally, we have identified two Rho-dependent terminators within the *flgL* sequence, T4_Rho and T5_Rho (Supplementary Fig. S3, and Supplementary Table S6), which may inhibit gene transcription. For further analysis, we also took the *oppA* gene coding for periplasmic binding protein OppA - an essential component of the oligopeptide transporter Opp. Importantly, in this case, the binding energy is also high (-18.5 kcal/mol), but this target did not appear in the RNA-Seq or proteomic analyses results.

To test if UpRoi1 RNA binds to its predicted basepairing site in mRNA *flgL*, we first used a gelshift assay (Fig. 7A, B, C). In these experiments, we used an 85-nt long mRNA fragment of the *flgL* gene. The data showed that UpRoi1 RNA forms a single complex with the 85-nt long fragment of *flgL* mRNA. UpRoi1 RNA binds *flgL* mRNA fragment with tight, low

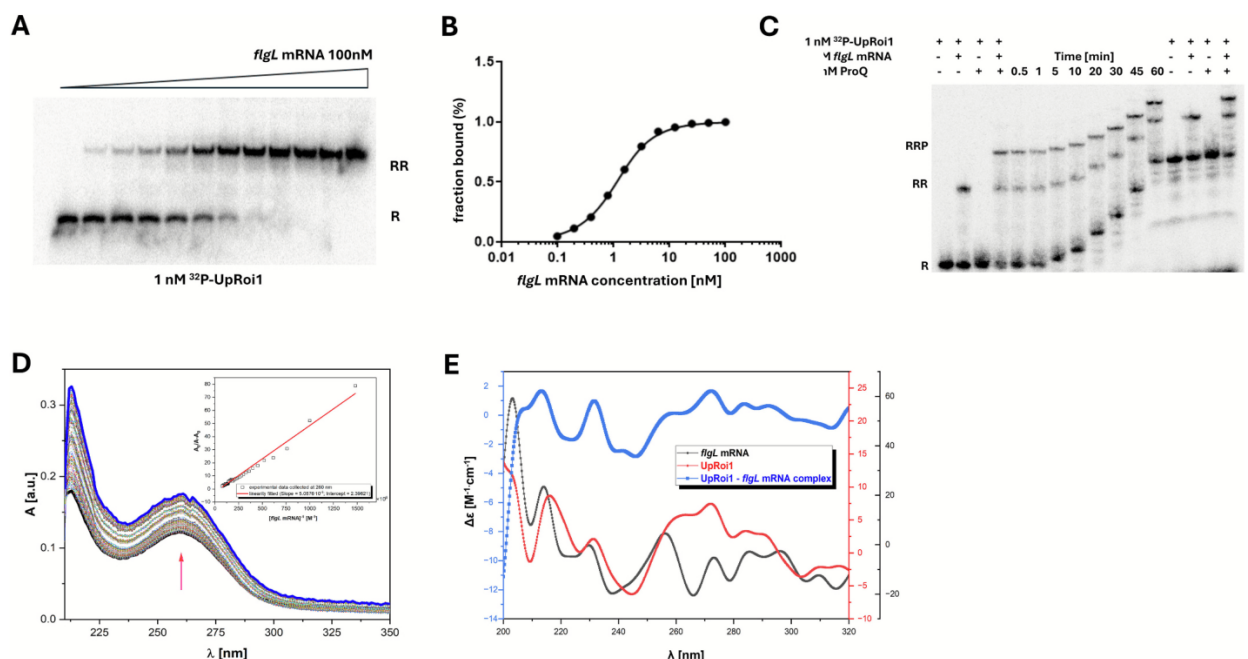


Fig. 7. The direct interaction of UpRoi1 with *flgL* mRNA and ProQ protein assessed by molecular and spectroscopic methods. (A) The pairing of the UpRoi1 sRNA to the *flgL* mRNA, monitored using a gelshift assay. Unbound ^{32}P -UpRoi1 sRNA is marked as R, and sRNA-mRNA complex as RR. (B) The fraction-bound data from panel A, plotted versus RNA concentration. (C) The association of ^{32}P -labeled UpRoi1 RNA to 85-nt long fragment of *flgL* mRNA in the presence of the ProQ protein. The binding was monitored using a gelshift assay. The control reactions are located on the left and right sides of the time course of binding, and the composition of each control reaction is noted above the gel. The unbound ^{32}P -UpRoi1 is marked as R, the complex of the *flgL* mRNA with ^{32}P -UpRoi1 RNA as RR, the complex of the 5'-UTR of *roi* mRNA with ^{32}P -UpRoi1 RNA and ProQ protein as RRP. (D) Absorption spectra of UpRoi1 (0.33 μM ; 0.5 ml) in the presence of increasing amounts of *flgL* mRNA solution (0.025 μM). The direction of the arrow indicates the hyperchromic effect resulting from intermolecular interactions. Inset: plot of $A_{250}/(A_{250} - A_{260}) = f(1/[flgL \text{ mRNA}])$ established as a result of interactions studied in Tris buffer (pH 7.5). (E) Circular dichroism (CD) spectra of the adequate RNA molecules UpRoi1 in red (0.33 μM), *flgL* mRNA in black (0.025 μM), and their complex in blue (0.16 μM). All assays were performed in three independent replicates.

nanomolar affinity. The gelshift assay also revealed that ProQ binds to the complex of *flgL* mRNA and UpRoi1 sRNA (RRP complex in Fig. 3C). The tight binding of UpRoi1 to *flgL* mRNA was further supported by UV spectrophotometric measurements and CD spectroscopy (Fig. 7D, E). In contrast, we did not observe the binding between UpRoi1 and *oppA* mRNA fragment (data not shown), thus this pair was not further analyzed.

UV electronic spectra revealed that increasing the concentration of *flgL* mRNA during the titration of the UpRoi1 solution led to a decrease in the energy of the $\pi \rightarrow \pi^*$ transition, which resulted in the appearance of a hyperchromic effect in the absorption spectra of UpRoi1. During the formation of the sRNA-mRNA complex, visible irregularities in absorbance increase were observed, despite maintaining a constant volume of the *flgL* mRNA titrant during measurement (Fig. 7D). Based on the analysis of data obtained from spectrophotometric titration, the binding constant ($K_{bind} = 4.71 \cdot 10^7 \text{ M}^{-1}$) was determined for the UpRoi1 – *flgL* mRNA complex (dependency in the upper right corner in Fig. 7D), describing the strength of interaction between these molecules as a derivative of the equilibrium of the association based on chromic effects

observed and by using Eq. (1) (see Section 4, Materials and methods) with a good linearity correlation parameter ($R^2 = 0.98232$; 298 K). Additionally, the value of Gibbs free energy ΔG was determined (-43.75 kJ/mol) by using Eq. (2), also presented in Section 4. Due to the above, the spontaneity of the binding reaction and the stability of the resulting complex were shown. Both parameters are interconnected and help assess the effectiveness of molecular interactions in tested biological environments.

Useful information for analysing the interaction between the two studied molecules was also obtained from the analysis of the absorption bands of the CD spectra registered in the range of 200–320 nm. The spectra of the individual RNA strands exhibited characteristic features typical of single-stranded RNA molecules. However, the spectrum recorded for the UpRoi1-*flgL* mRNA complex clearly differed from the simple algebraic sum of the spectra of the two RNA strands. This deviation indicates that the two strands interact directly and that their conformational arrangements change upon complex formation. The observed hypochromic effects and slight hypochromic shifts (Fig. 7E), particularly in the regions around 225 and 265 nm, indicate the

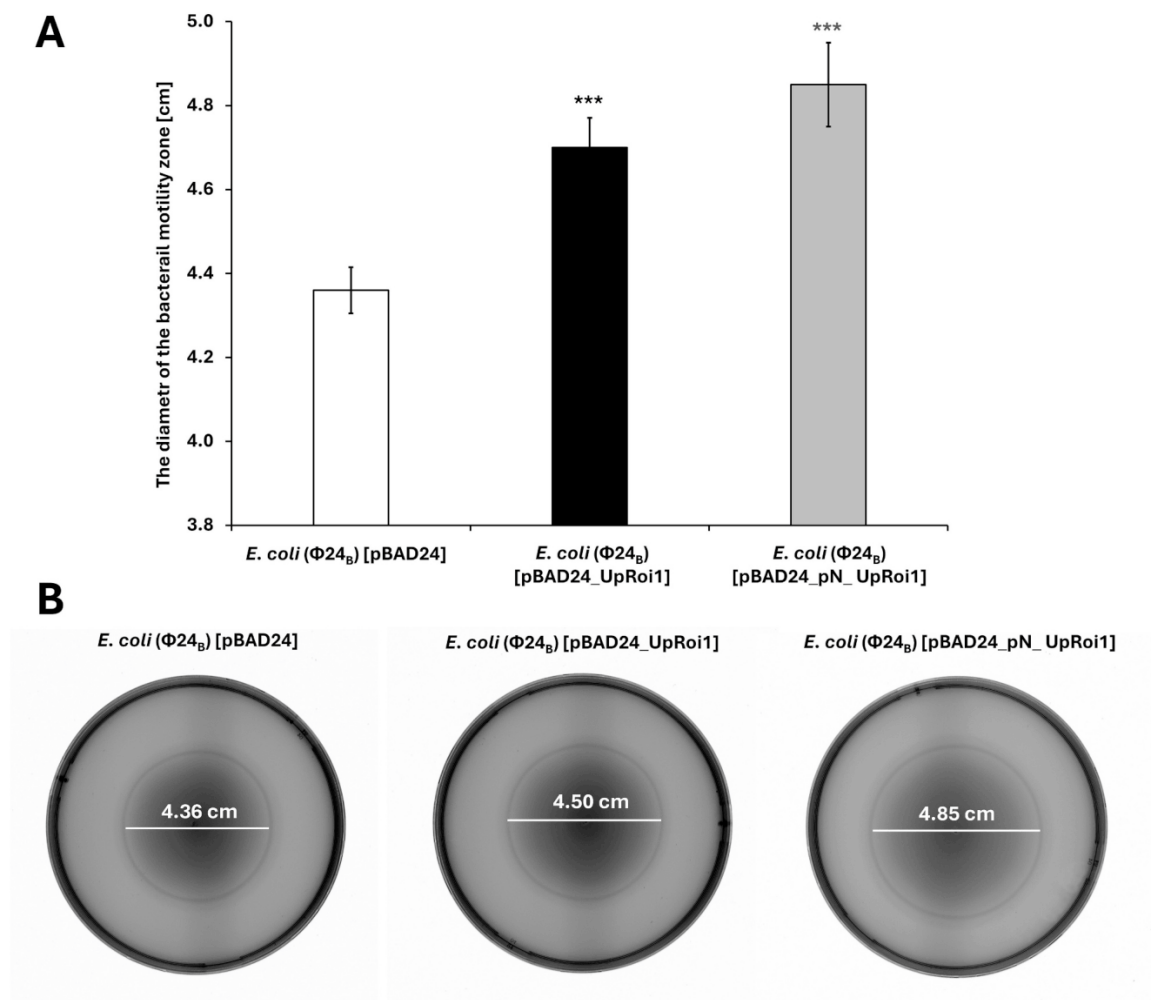


Fig. 8. Motility assay of the *E. coli* (Φ24_b) cells bearing plasmid pBAD24, pBAD24_UpRoi1, or pBAD24_pN_UpRoi1. The data set was generated for samples withdrawn at 60 min after dilution under spontaneous induction conditions (no mitomycin C was added). (A) Graph displaying the migrated distances (in cm) of each *E. coli* strain. The *p* values are marked with stars (***) for $p < 0.001$ and indicate statistically significant differences between tested variants and the control *E. coli* (Φ24_b) [pBAD24] strain. The error bars present the standard deviation of the biological triplicate. (B) Representative soft agar plates showing motility zones measured in centimetres (cm).

formation of new interstrand interactions and partial reorganization of base stacking. Hypochromism is generally caused by enhanced π - π interactions between adjacent bases, which reduce the overall transition dipole moment, whereas blue shifts suggest a more compact or rigid helical arrangement. These spectral changes reflect a rearrangement of the secondary structure, most likely associated with hybridization-like base pairing or partial duplex formation between complementary sequence regions of the two RNAs. In addition, the disappearance or reduced intensity of certain CD bands compared to the free RNA spectra suggests that complex formation also affects the global helical twisting and backbone conformation. Such alterations are typical of RNA-RNA interactions, where structural elements, such as loops, bulges, or single-stranded fragments, undergo local reorganization to accommodate the second strand. Overall, the CD data confirmed that the interaction between UpRoi1 and *flgL* mRNA involves not only electrostatic recognition but also specific structural adjustments, leading to a more ordered, partially double-helical complex.

Finally, we tested if UpRoi1-mediated regulation of the bacterial host gene expression might have any significant effect on *E. coli* physiology. Since the *flgL* gene, coding for a protein involved in the formation of the junction between the flagellar hook and the filament, was up-regulated in the UpRoi1-overproducing variant analyzed by RNA-seq and RT-qPCR methods (Fig. 5D, Supplementary Tables S4 and S5), we decided to analyze the efficiency of motility of *E. coli* cells overexpressing (or not) the *uproi1* gene. We observed that the swarming phenotype, which reflects the efficiency of motility of bacterial cells on agar nutrient plates, was considerably more pronounced in cells overproducing UpRoi1 from its natural *pN* promoter than in cells from other tested variants (Fig. 8). The analysis was performed under conditions of spontaneous induction, as no inducer of the lytic cycle was added to the culture.

These results indicated that the small, Φ 24_B phage-encoded, microRNA-like UpRoi1 molecule has significant effects on the expression of the *E. coli* host genes, and on some physiological processes.

3. Discussion

While small regulatory RNAs encoded by bacteriophages that influence the switch between lytic and lysogenic cycles have obtained little attention up to now [14–16], similar molecules have been well-known in viruses that infect eukaryotic cells, particularly herpesviruses [17,18]. A closer examination of these viruses reveals certain parallels with tailed bacteriophages (*Caudovirales*), a group that includes Stx phages. Some researchers have even suggested that herpesviruses and *Caudovirales*, both large double-stranded DNA viruses, share structural and evolutionary connections [19,20], as well as similarities in their life cycles. Like Stx phages, herpesviruses can alternate between two infection states, a latent phase and a lytic phase. In both cases, latency or lysogeny represents a persistent form of infection in which most viral genes remain silent, with only a selected few being expressed to help evade

host defenses. When reactivated, herpesviruses start the lytic cycle, destroying the host cell and releasing viral progeny that spreads in the environment [21,22].

Herpesvirus-encoded small regulatory RNAs, particularly microRNAs, play a key role in regulating the virus life cycle transition [17]. The prevailing model of microRNA-mediated regulation suggests that during latency, microRNAs are abundantly expressed and work to maintain this state by suppressing lytic gene expression [17,23]. Notably, some viral microRNAs have been found to promote lytic infection rather than inhibit it [24–26]. Additionally, several of these microRNAs originate from the antisense strand of viral genes, exhibiting perfect complementarity to their target mRNAs [27]. Among those, the role of viral microRNAs in facilitating viral persistence and tropism by targeting genes associated with many cellular processes (e.g., apoptosis, angiogenesis, cell migration, etc.) was also reported [28].

In general, *cis*-encoded antisense small RNAs influence gene expression by forming RNA duplexes, which can alter ribosome binding/translation, transcription termination, or mRNA stability by modifying secondary structures such as hairpins [29,30]. Given these findings and the many similarities between herpesviruses and Stx phages, it was crucial to determine whether a similar microRNA-based regulatory mechanism exists in temperate bacteriophages. We have previously demonstrated that the Stx phage-derived 24B_1 molecule, similar in size to eukaryotic microRNA, favors the lysogeny state, and this was the first evidence of the occurrence of such regulation in bacterial viruses [14,15]. In this work, we proved that another small (30-nt long) phage-encoded RNA, called UpRoi1, indeed plays a pivotal role in deciding between lysogeny and lysis. This discovery may significantly shift our understanding of phage development, as the role of RNA-RNA interactions during lytic activation of temperate phages is still poorly understood. Besides, following the findings of Sprenger et al. [13] and Altuvia et al. [12], our results provide further evidence that small non-coding RNA molecules have a significant impact on phage and its bacterial host interaction and may be a part of phage-encoded quorum sensing (QS)-like communication systems.

The proposed mechanism of regulation of the Stx phage Φ 24_B life cycle switch, from lysogenic to lytic mode, dependent on *cis*-acting UpRoi1 sRNA, starts with the LexA protein (Fig. 9). As mentioned above, LexA is a repressor of the SOS regulon whose activation is also important for the Stx prophage induction. The inducers of the SOS response (e.g. UV irradiation or some antibiotics, such as mitomycin C) lead to DNA damage, activating the RecA protein, which subsequently facilitates the degradation of the bacterial LexA protein [31]. The direct interaction of LexA repressor with the *pN* promoter of the *uproi1* gene, proven in this work (Fig. 4), may suggest its inhibitory influence on this gene expression. According to our proposal, during the SOS response, when the LexA protein is inactivated by active RecA protein, the *pN* promoter is released from the inhibitory action of this repressor. In effect, the *uproi1* gene is transcribed, and mature UpRoi1 stimulates the production of Roi antirepressor.

Importantly, RecA activation (due to DNA damage) may actually trigger two complementary mechanisms of *cI* inactivation: one through direct autocleavage of *cI* protein (as discussed by Licznarska et al. [31] and above), and the other indirect through degradation of LexA and thus unblocking transcription of the antirepressor gene, whose product competes with *cI* for binding to operator sites in the *pR* promoter from which the transcription of early phage genes of lytic cycle starts. The second mechanism has been well-described in the case of the Cro antirepressor in lambda phage, which occupies the operator site and prevents RNA polymerase from initiating transcription from the *cI* gene promoter. In this case, Cro antirepressor inhibits the synthesis of *cI* mRNA and thus complements the first mechanism based on autolysis of the produced *cI* proteins [31]. As can be seen in the example of lambda phage, both regulation mechanisms can function in the cell and complement each other to control the switch from lysogenic to lytic phase cycle. Such a stringent regulation of the lytic cycle is crucial

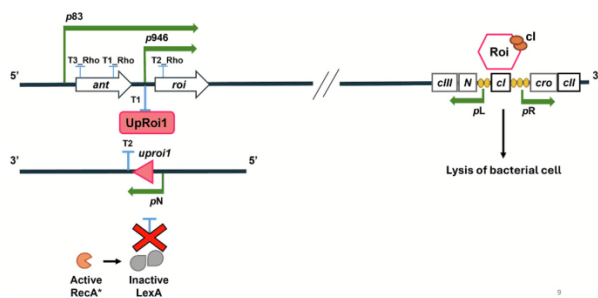


Fig. 9. The proposed mechanism of the UpRoi1 sRNA-mediated switch between lysogenic and lytic phage development (described in detail in the text).

because, on the one hand, it allows the phage to produce progeny, but on the other, it leads to the death of the host, on which the phage is 100% dependent. For this reason, phages have evolved mechanisms that limit their lytic development to a certain number of cells within the bacterial population. Moreover, the efficiency of prophage induction depends on the applied inductor. For example, hydrogen peroxide, a natural inducer, induces the phage lytic cycle in only 1% of the bacteria in the population [31]. For comparison, a low concentration of mitomycin C (up to 1 µg/ml) was reported to initiate lytic development in about 10–30% cells in the bacterial population [31]. Importantly, even without an applied specific inducer, spontaneous induction still occurs in a small percentage of bacteria in the population.

In this work, we proved that the UpRoi1 molecule binds, based on full complementarity, to the region with overlapping sequences of the promoter *p946* and T1 terminator, located within the 5'UTR region of the *roi* gene (Figs. 1 and 3, Supplementary Fig. S2). As a result of such binding, the secondary structure of the T1 terminator may unravel, thus enabling the transcription of *roi* from the *p946* promoter. On the other hand, the action of UpRoi1 may also negatively affect the binding of the Rho protein to its utility site (*rut*), which is located upstream of the *roi* start codon (Fig. 9, Supplementary Fig. S2, and more details below). In effect, there is no transcription termination at the T1_Rho terminator region, and expression of *roi* (as a continuation of transcription from the *p83* promoter) may occur. Curiously, there is an additional *rut* site located downstream of the *roi* start codon. This site is located within the T2_Rho terminator that may inhibit the transcription from the *p946* promoter. Due to the UpRoi1 action, this site also might be less accessible for Rho protein. As a result of UpRoi1-mediated antitermination, the expression of the *roi* gene increases, which we observed in the RNA-seq and RT-qPCR data (Supplementary Table S4 and Fig. 5D). In effect of such a regulation, the antirepressor protein Roi is produced and counteracts the *cI* repressor action, thus enabling the phage to enter the lytic development path, which is manifested by the decreased number of live bacteria in the culture, caused by cell lysis (Fig. 2).

The mode of action of UpRoi1 resembles that of the herpesviral microRNAs miR-200b and miR-429, which promote EBV lytic replication by targeting ZEB1 and ZEB2 and thereby relieving repression of the Zp promoter of BZLF1, a key trigger of the latency-to-lytic switch [24,25]. Moreover, the regulation of the expression of phage anti-repressor genes by bacterial sRNA is also known. For example, the antirepressor Ant synthesis is regulated during the development of bacteriophage P22 by the small antisense RNA named Sar. Notably, this sRNA molecule acts *in trans* by binding to the ribosome binding site of Ant mRNA and inhibits Ant production at a post-transcriptional level [9,32]. Similarly, in phage N15, which is a temperate virus of *Escherichia coli* related to lambdoid phages, the antirepressor gene is negatively regulated by a small RNA, named CA RNA [33]. Interestingly, contrary to the phage UpRoi1 molecule, the indicated bacterial sRNAs negatively regulate the expression of the antirepressor genes.

On the other hand, similarly to microRNAs of herpesviruses EBV and Kaposi's Sarcoma-associated Herpesvirus (KSHV), the phage UpRoi1 sRNA may also affect the migration of bacterial host cells through direct binding to *flgL* mRNA and its up-regulation, together with up-regulation of other flagellar-associated genes *flgK*, *fljD*, *flgM*, and *flgN* (Supplementary Table S4, Figs. 5D, 7 and 8). The FlgL protein, encoded by the *flgL* gene, plays a crucial role in connecting the hook to the filament in the bacterial flagellum. Working alongside FlgK, it establishes the structural foundation necessary for the attachment of flagellin, the protein responsible for filament formation and elongation. The absence of either FlgL or FlgK disrupts flagellum assembly, resulting in the appearance of bacteria that are non-motile due to their inability to produce functional flagella [34].

The direct binding between UpRoi1 and *flgL* mRNA molecules was proven by a classical gelshift assay (Fig. 7A–C) and two other methods: UV spectrophotometric measurements and CD spectroscopy (Fig. 7D, E). The high binding constant (K_{bind} is $4.71 \cdot 10^7 \text{ M}^{-1}$) of UV data (Fig. 7D)

reflects complex stability attributed to longer precursor sequences and additional hydrogen bonds [35–38]. The calculated Gibbs free energy (-43.75 kJ/mol) supports this conclusion, aligning with RNA duplex predictions [39]. On the other hand, the CD spectrum of the complex (Fig. 7E) closely resembles that of *flgL* mRNA, implying it serves as the template for UpRoi1 binding. Minimal band shifts suggest that *flgL* mRNA retains its conformation, while significant intensity changes at 207 nm indicate reduced base stacking and RNA aggregation upon binding [40–43]. The direct regulation of *flgL* by UpRoi1 was further confirmed by phenotypic effect, as the swarming phenotype, which reflects the efficiency of bacterial cells' motility, was also proven to be considerably more pronounced in cells overproducing UpRoi1 than in control cells (Fig. 8). Interestingly, this effect is also observed in the pBAD24_UpRoi1 variant, in which a small number of UpRoi1 molecules may be produced as a result of pBAD promoter leakage.

Because phage proteins are likely required for UpRoi1 molecule production, we could not perform experiments under lysogen-free conditions, and have performed this assay under spontaneous induction conditions, where prophage induction occurred at a low level and only in a small fraction of cells in the population. Our results indicated that overproduction of the UpRoi1 molecule affected the motility of the remaining cells in the population, which have not been lysed. We suspect that even a small number of UpRoi1 molecules is sufficient to induce this effect, and most likely, UpRoi1 molecules released as a result of cell lysis affected other cells in the population where prophage induction has not yet occurred. The cells became more motile, allowing them to travel greater distances to achieve optimal phage growth conditions. This is important because it would not be profitable for phages to kill all bacterial cells in an infected population. They sacrifice some bacteria to multiply, but not all, as without them, they could not propagate. Phages, like other biological entities, evolved to maintain their genetic material, so they preserve some cells in good condition and stimulate them to seek optimal conditions for their own growth and development. It appears that the UpRoi1 molecule may act as a quorum regulatory sRNA and play a role in cellular communications.

Interestingly, similar effects have been reported for eukaryotic viruses, where herpesviral microRNAs promote host cell migration, invasion, angiogenesis, contributing to the dissemination of virus-induced tumors [28]. Based on these findings, we propose that the UpRoi1-mediated regulation of bacterial motility ensures the appropriate dissemination of new phage particles released from the cell during the lysis process.

Bacteria commonly adjust the expression of flagellar components based on environmental conditions. When nutrients are scarce [44] or temperatures are unfavorable [45], motility is often suppressed to save energy for processes essential for survival. Importantly, the regulation of cell motility in response to changes in nutrient availability can also be controlled by sRNAs. The example is McaS sRNA that activates *flhD*, the master transcription regulator of flagella synthesis, leading to increased motility of *E. coli* [44]. Based on these findings, we propose that through the action of UpRoi1, the phage may develop a similar mechanism, by enhancing the *flgL* gene expression and further the synthesis of flagellar protein FlgL. As a result, the bacterial cell could be compelled to move toward a more favorable environment – (I) with abundant nutrients as nutrient deficiency inhibits phage replication or (II) an appropriate concentration of bacterial hosts since high bacterial density induces production of the *quorum sensing* signals, and may decrease phage production [46].

Based on the above-mentioned UpRoi1-dependent positive regulation of *roi* and *flgL* gene expression, we speculate that UpRoi1 may regulate gene expression by acting on terminators and reducing the transcription termination. Importantly, the sRNA-mediated termination abolition is well-known in bacteria. Sedlyarova et al. [47] showed that sRNAs DsrA, ArcZ, and RprA bind the 5'UTR of the *rpoS* gene to suppress premature Rho-dependent transcription termination and markedly stimulate its expression. In our findings, such a target of the termination

inhibition action of the UpRoi1 molecule might be the T1_Rho terminator or T2_Rho terminator located, respectively, upstream or downstream of the *roi* start codon (Fig. 1C and Supplementary Fig. S2). Other targets could be the T4_Rho and T5_Rho terminators found within the coding sequence of the *flgL* gene (Supplementary Fig. S3 and Supplementary Table S6). Essentially, Rho plays a crucial role in silencing transcription, primarily influencing the 5'UTRs of numerous bacterial genes, thus, its inhibition by small RNAs represents a widespread mode of bacterial gene regulation. Farley and collaborators [48] proposed a model for *cfa* regulation by Rho and both repressing and activating sRNAs that increased or reduced transcription termination efficiency, respectively. Interestingly, the sRNA-dependent antitermination, in this case, was mediated by RydC sRNA whose binding site was located downstream of the Rho utilization site (*rut*) and whose action made the *rut* less accessible to the Rho protein. An important role in this regulation is played by an RNA chaperone, the Hfq protein. A similar observation that RNA chaperones frequently assist the sRNA-mediated antitermination of transcription was reported by Sedlyarova et al. [47]. This, together with our findings showing the binding of ProQ protein to the sRNA-RNA complex (Figs. 3 and 7), allow us to suspect that this RNA-specific chaperone protein may participate in the UpRoi1-mediated antitermination of the gene transcription.

Considering the small size of UpRoi1, its ability to act in both *cis* and *trans* as well as the high number of identified potential target sites for this molecule in both phage and bacterial genomes, accompanied with similar functions to herpesvirus microRNAs, we suggest that the UpRoi1 may belong to analogous group of small non-coding RNAs in prokaryotes. Among the potential target genes, identified but not further analyzed in this work, whose expression may be regulated by the UpRoi1 molecule, *cspG* and *narP* seem worthy of attention. The first gene encodes cold-shock protein, and we observed a significantly decreased level of this protein in proteomic analysis (Fig. 6). Besides, the IntaRNA software predicted strong energy hybridization (−19.4 kcal/mol) of *cspG* mRNA binding to the UpRoi1 molecule (Supplementary Table S2). Importantly, the reduction of the mitochondrial heat shock protein A9 (HSPA9) level by herpesviral (KSHV) microRNAs, which in effect decreased mitochondrial copy numbers and enhanced anaerobic glycolysis (Warburg effect), was also reported [49,50]. Curiously, phage 24B_1 sRNA also affects this process [15]. The second gene, *narP*, encodes a protein belonging to a two-component system that in response to nitrate and nitrite, regulates the expression of genes involved in anaerobic respiration. Interestingly, as reported previously, NarP synthesis is negatively regulated by two sRNAs, SdsN137 and RprA [51], and its mRNA shows high hybridization energy with UpRoi1 (−14.4 kcal/mol) as indicated in Supplementary Table S2. Although the *narP* gene was not included in the list of differentially expressed genes generated by RNA-seq, the two-component system was indicated among the most affected processes in the KEGG pathway enrichment analysis (Fig. 5). Apart from that, an interesting observation is also a slightly increased expression of the genes encoding the remaining two antirepressors, Ant and D_{ant}, in the variant of UpRoi1 overproduction (Supplementary Table S4 and Fig. 5D). Since we did not find binding sites for the UpRoi1 molecule within *ant* and *d_{ant}* genes, we speculate that the observed increase of their expression may be an effect of the indirect action of the UpRoi1 molecule. We also assume a similar indirect regulation of genes encoding products related to nucleic acids metabolism and ribosomal activity (Fig. 6), as bioinformatic analysis did not confirm the presence of the UpRoi1 binding sites within their sequences. Interestingly, the report of McCool et al. [52] indicated that in higher eukaryotes, the ribosome biogenesis process is regulated at multiple steps by microRNAs.

Based on the above findings and considerations, we assess the UpRoi1 molecule as similar to herpesviral microRNAs in terms of its functions and role in the development of the virus and its host. Viral microRNAs in eukaryotic systems are known to regulate host gene expression, influencing the switch between latent and lytic phases and facilitating immune evasion. Given their similar roles in phage-bacteria

interactions and comparable structural properties, phage-derived sRNAs, like UpRoi1, may function similarly in modulating host and phage genes through *cis*- or *trans*-acting. The importance of this regulation is demonstrated by the increasing number of reported examples of bacterial processes controlled by phage sRNAs [53]. These findings highlight the critical role of sRNAs in regulating phage-host dynamics and suggest that targeting these regulatory RNAs could provide a new avenue for therapeutic intervention. By disrupting the interactions between sRNAs and their targets, it may be possible to maintain phages in the lysogenic state, thereby minimizing Shiga toxin production and reducing the risk of severe disease outcomes in STEC infections. On the other hand, demonstrating similarities between herpesviral microRNAs and UpRoi1 is of great importance, because it justifies the occurrence of microRNA-type molecules in Stx phages.

4. Materials and methods

4.1. Bacterial strains and growth conditions

E. coli MC1061 [54], *E. coli* MG1655 [55], and their derivatives bearing the Φ 24_B prophage (Δ stx2::cat [56]) were employed in this work. For all experiments, *E. coli* liquid cultures were grown in Luria-Bertani (LB) medium (BioShop, Burlington, ON, Canada) with aeration at 37 °C in a shaking incubator (200 RPM). The LB broth supplemented with 1.5% agar (BTL, Łódź, Poland) was used as a bottom agar. The plates with solid medium were incubated at 37 °C for 16–18 h. Ampicillin (BioShop, Burlington, ON, Canada) was added to final concentrations of 50 µg/ml, when required.

4.2. Propagation of bacteriophages

E. coli MC1061 lysogenic with bacteriophage Φ 24_B was grown in LB medium at 37 °C to an OD₆₀₀ of 0.1. The prophage induction process was provoked by adding mitomycin C (BioShop, Burlington, ON, Canada) to a final concentration of 1 µg/ml. The cultivation was continued at 37 °C with shaking until bacterial cell lysis occurred. The lysate was treated with 4% chloroform for 15 min (Chempur, Piekary Slaskie, Poland) and then centrifuged (2000 ×g, 10 min, 4 °C). For phage particles concentration, 10% polyethylene glycol (PEG) 8000 (BioShop, Burlington, ON, Canada) was added to the supernatant. The mixture was stirred overnight at 4 °C. In the next step, the precipitate was collected by centrifugation (8000 ×g, 20 min, 4 °C), and the phage pellet was suspended in TM buffer (10 mM Tris-HCl, 10 mM MgSO₄; pH 7.2). To remove PEG 8000, the sample was treated three times with chloroform and centrifuged (2000 ×g, 10 min, 4 °C). The upper phase with virus particles was collected and diluted 10-fold in the TM buffer. Then, 2.5 µl of each dilution of phage lysate was spotted onto a freshly prepared double-layer agar in Petri dishes.

The top layer was prepared by mixing 1 ml of the overnight *E. coli* MC1061 culture with 2 ml of 0.7% bacteriological agar (BTL, Łódź, Poland), supplemented with 10 mM MgSO₄ (Chempur, Piekary Slaskie, Poland) and 10 mM CaCl₂ (Chempur, Piekary Slaskie, Poland). Then, the mixture was poured onto 1.5% solid LB agar with a sublethal concentration of chloramphenicol (BioShop, Burlington, ON, Canada), according to the previous procedure [57]. After the plates' overnight incubation at 37 °C, the plaques were counted, and the phage titer (PFU/ml) was quantified. The phage lysate was stored at 4 °C until use in subsequent experiments.

4.3. Searching for new microRNA-type molecules less than 50 nt in length using next-generation sequencing (NGS)

To identify the fraction of very small RNA molecules (<50-nt long) in bacterial cells, the procedure described earlier [58] was employed. Briefly, *E. coli* MG1655 lysogenic with phage Φ 24_B was grown in LB medium at 37 °C to an OD₆₀₀ of 0.1. In the next step, bacterial cells were

treated with 0.5 µg/ml mitomycin C (BioShop, Burlington, ON, Canada) to induce the prophage. At specified times, 5×10^8 *E. coli* cells were withdrawn and centrifuged in a microcentrifuge (2000×g, 5 min, room temperature). The obtained pellets were frozen in liquid nitrogen and then treated with TRIzol™ Reagent (Thermo Fischer Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instructions. After bacterial cell lysis, small RNA molecules were isolated using the Ambion™ PureLink™ miRNA Isolation Kit (Thermo Fischer Scientific, Waltham, Massachusetts, USA) as described in the manual. Genomic DNA from the samples was digested with the TURBO DNA-free™ Kit (Thermo Fischer Scientific, Waltham, Massachusetts, USA). The quantity and quality of isolated small RNAs were assessed by a NanoDrop OneC spectrophotometer (Thermo Fischer Scientific, Waltham, Massachusetts, USA) and the Qubit™ microRNA Assay Kit. Then, the purified sRNA molecules were concentrated by the standard precipitation method using 100% or 80% ethanol (Avantor Performance Materials Poland S.A., Gliwice, Poland), 3 M sodium acetate (pH 5.3; BioShop, Burlington, ON, Canada), and 100 µg of glycogen (Thermo Fischer Scientific, Waltham, Massachusetts, USA). In the next step, the fraction of sRNAs less than 50 nt in length was recovered from the 15% polyacrylamide gel (19 acrylamide: 1 bis-acrylamide; Merck KGaA, Darmstadt, Germany) with 7 M urea (Avantor Performance Materials Poland S.A., Gliwice, Poland) by using Urea-PAGE and the electroelution method. The eluted sRNAs were concentrated using the standard precipitation method as described above, and then the sRNAs library was generated using the SMARTer smRNA-Seq Kit for Illumina® (TAKARA BIO INC., Kusatsu, Prefektura Shiga, Japan) according to the procedure presented in the provider's protocol. Following reverse transcription, sequencing of cDNA libraries was conducted with the NexSeq 500 Illumina Sequencer.

4.4. Plasmids' construction

Plasmid pBAD24 (Gentaur, Sopot, Poland) was employed as a vector and control variant in all experiments presented in this work. Constructions of pBAD24 derivatives were performed according to the standard methods of molecular cloning [59]. The genomic DNA of *E. coli* MC1061 lysogenic with bacteriophage $\Phi 24_B$ was isolated by using the NucleoSpin® Tissue kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany), according to the manufacturer's protocol. Specific oligonucleotide primers were designed by Primer3web (version 4.1.0) and synthesized by GENOMED S.A. (Warsaw, Poland). The construction of plasmid pBAD24 derivatives was confirmed by DNA sequencing (GENOMED S.A., Warsaw, Poland), using primers: F_pBAD24 (5' ATGCCA-TAGCATTTTATCC 3') and F_pBAD24 (5' GATTTAATCTGTATCAGG 3').

For construction of the plasmid pBAD24_UpRoi1, the region from phage $\Phi 24_B$ DNA (GenBank: HM208303; complement strand – from 19200 to 19439) encompassing the 30-nt long nucleotide sequence of UpRoi1 molecule was amplified by PCR primers F_ $\Phi 24_B$ _UpRoi1_EcoRI (5' CATGAATTCACACTTTTGCATTGAAGGAAGT 3') and R_ $\Phi 24_B$ _UpRoi1_HindIII (5' CCTAAGCTTTCACTGCATCGCTACTGACA 3'). Following digestion with EcoRI and HindIII restriction endonucleases (Thermo Fischer Scientific, Waltham, Massachusetts, USA), the PCR-amplified DNA was ligated with the EcoRI-HindIII fragment of plasmid pBAD24 bearing an ampicillin-resistant gene and the pBAD promoter of the araBAD (arabinose) operon [60] by using the T4 DNA ligase (Thermo Fischer Scientific, Waltham, Massachusetts, USA). After the transformation of competent MC1061 *E. coli* cells with the plasmid pBAD24_UpRoi1, bacteria were frozen in 15% glycerol and stored at –80 °C.

The plasmid pBAD24B_pN_UpRoi1 was constructed according to a similar procedure. To amplify a DNA fragment containing the nucleotide sequence of $\Phi 24_B$ genome (GenBank: HM208303; complement strand – from 19200 to 19467) encompassing the 30-nt long nucleotide sequence of UpRoi1 molecule with its bioinformatically predicted promoter pN by PCR method, two primers: F_pN_UpRoi1_EcoRI (5'

CGATGAATCTTGTAGGGCTAATGTTTGT 3') and F_UpRoi1_HindIII (5' CCTAAGCTTTCACTGCATCGCTACTGACA 3'), were used. In the next step, the amplified insert and vector pBAD24 were cut with EcoRI and BamHI enzymes (Thermo Fischer Scientific, Waltham, Massachusetts, USA) and ligated by the T4 DNA ligase (Thermo Fischer Scientific, Waltham, Massachusetts, USA), following the instructions supplied by the provider. After the transformation of competent MC1061 *E. coli* cells with the plasmid pBAD24_pN_UpRoi1, bacteria were frozen in 15% glycerol and stored at –80 °C.

4.5. Detection and quantitation of 30-nt long sequence of UpRoi1 small RNA by RT-qPCR

E. coli MC1061 ($\Phi 24_B$) cells bearing plasmid pBAD24, pBAD24_UpRoi1 or pBAD24_pN_UpRoi1 were grown in LB liquid medium at 37 °C with aeration to an OD₆₀₀ of 0.1. In the next step, each of the bacterial cultures was divided into two aliquots. One of them was treated with mitomycin C (1 µg/ml; BioShop, Burlington, ON, Canada) to induce the lytic cycle of the $\Phi 24_B$ prophage. The second one was a control without an induction agent. At the appropriate time points, 5×10^8 bacterial cells were harvested. To inhibit the growth of bacteria, the samples were treated with 10 mM sodium azide (VWR International, Radnor, Pennsylvania, USA), centrifuged (2000 ×g, 10 min, 4 °C), and then the obtained sediments were deep-frozen in liquid nitrogen for 30 s. sRNAs from all samples were isolated with the Ambion™ PureLink™ miRNA Isolation Kit (Thermo Fischer Scientific, Waltham, Massachusetts, USA). To remove DNA from sRNA preparations, the TURBO DNA-free™ Kit was employed (Thermo Fischer Scientific, Waltham, Massachusetts, USA). The quality and quantity of isolated sRNAs were examined using a NanoDrop OneC spectrophotometer (Thermo Fischer Scientific, Waltham, Massachusetts, USA). Detection and quantification of UpRoi1 sRNA expression through a poly(A) sRNA-based real-time PCR approach were conducted as described previously [15,61], with some modifications. Briefly, 200 ng of isolated sRNA was polyadenylated with ATP by poly(A) polymerase at 37 °C for 30 min in a 30 µl reaction mixture, following the manufacturer's recommendations (EURx Sp. z o. o., Gdansk, Poland). After polyadenylation of 3' termini of sRNAs, cDNA from the polyadenylated sRNA samples (100 ng) was obtained with Transcriptor Reverse Transcriptase (Roche Diagnostics International, Rotkreuz, Switzerland) and poly(T) adapter primer (5' GCGAGACAGAATTAATACGACTCACTATAGG(T)12VN 3'; GENOMED S.A., Warsaw, Poland), according to the instructions supplied from the provider. Analysis of expression of UpRoi1 microRNA-tape molecule by RT-qPCR in all tested samples was performed using LightCycler® 480 Real-Time PCR System (Roche Diagnostics International, Rotkreuz, Switzerland). All reactions were prepared in 96-well plates containing 10 µl of 2× SYBR Green I Master Mix (Roche Diagnostics International, Rotkreuz, Switzerland), 7 ng cDNA, and 500 µM specific primers. The reverse primer was a 3' universal adapter primer (5' GCGAGACAGAATTAATACGAC 3'; GENOMED S.A., Warsaw, Poland), and the forward primer was designed based on the entire tested UpRoi1 sequence (5' TGTCTCTCACATTGAAAATTCAG 3'; GENOMED S.A., Warsaw, Poland). Relative quantification assays were performed with cDNA relative to *icdA* housekeeping gene [62]. To determine the expression level of the *icdA* gene in tested samples, the previously described by us procedures and primers (pF_ *E. coli icdA* – 5' AGGCAAGAA-GATCACCCGTC 3' and pR_ *E. coli icdA* – 5' GCATGGCTGGGGTTACATCT 3'; GENOMED S.A., Warsaw, Poland) were employed [14,15]. Cycling conditions were 95 °C for 5 min followed by 55 cycles of 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 15 s. No-template controls and a melting curve analysis (60 °C) were performed to ensure the specificity of the amplification. The relative changes in UpRoi1 expression revealed by RT-qPCR were calculated using the calibrator normalized relative quantification method with efficiency correction (E-Method). PCR efficiency was determined for each reaction by the LinRegPCR program [14,15,63].

4.6. Kinetics of phage lytic development after prophage induction with mitomycin C

The prophage induction experiment was performed as described previously [64], with some modifications. Briefly, *E. coli* MC1061 (Φ 24_B) cells bearing plasmid pBAD24, pBAD24_UpRoi1, or pBAD24_pN_UpRoi1 were cultivated in LB medium at 37 °C to an OD₆₀₀ of 0.1. Then, bacterial cultures were diluted 100-fold in the LB medium. Phage lytic development was provoked in lysogenic host bacteria by adding mitomycin C (BioShop, Burlington, ON, Canada) at one of the following concentrations 0.25, 0.5, or 1 µg/ml. The one exception was spontaneous induction when no mitomycin C was added. The cultivation was continued with aeration at 37 °C. Bacterial growth after prophage induction was monitored by measuring OD₆₀₀ at 30 min intervals over the period of 360 min. The number of phage infective particles per ml (PFU/ml) was also determined during this experiment. To calculate the phage titer, samples were taken at 360 min, mixed with 6% chloroform (Chempur, Piekary Slaskie, Poland), and centrifuged (2000 ×g, 5 min, room temperature). The supernatant with the virus particles was diluted in TM buffer (10 mM Tris-HCl, 10 mM MgSO₄, pH 7.2) and spotted onto a double-layer agar plate according to the procedure described earlier [57]. After overnight incubation of the plates at 37 °C, the phage titer (PFU/ml) was estimated.

4.7. Bacteria enumeration after phage infection under nutrient-starvation conditions

The procedure described earlier was used to estimate the number of cells that survived after phage infection, with slight modification [14]. Briefly, host bacteria bearing plasmid pBAD24, pBAD24_UpRoi1, or pBAD24_pN_UpRoi1 were grown in LB medium at 37 °C to an OD₆₀₀ of 0.2. In the next step, 4 ml of the tested sample was centrifuged (2000 ×g, 10 min, 4 °C). The culture was washed three times with TCM buffer (10 mM Tris-HCl, 10 mM MgSO₄, 10 mM CaCl₂; pH 7.2), and then the obtained pellet was resuspended in 1 ml of the same buffer (pro-lysogenic conditions). Phage particles were added to the bacterial cells to a multiplicity of infection (m.o.i.) of 5. The TM buffer without virus particles was used in the control sample. Following incubation of the sample for 30 min at 37 °C, serial 10-fold dilutions of the mixture were prepared in 0.85% NaCl (Chempur, Piekary Slaskie, Poland). Then, 100 µl of each dilution was spread on LB agar plates with ampicillin (BioShop, Burlington, ON, Canada) at a final concentration of 50 µg/ml. After 20 h of incubation at 37 °C, the number of colony-forming units per ml (CFU/ml) was determined.

4.8. The efficiency of prophage formation in host bacteria under pro-lysogenic conditions

The number of lysogens after infection of host bacteria with bacteriophage was determined according to the procedure described earlier by [65], with some modifications. Briefly, all bacterial colonies that survived the viral infection and grew on a selected dilution were streaked separately onto LB agar supplemented with 20 µg/ml chloramphenicol (BioShop, Burlington, ON, Canada). Plates were incubated at 37 °C overnight. Because the gene of chloramphenicol resistance is located only in the prophage genome, bacterial colonies that grew on the selective medium were considered lysogens. The number of lysogens per ml (CFU/ml) was calculated.

4.9. RNAs preparation, transcription in vitro, and binding assay

A 30-nt UpRoi1 RNA (5' CAUGUCCUCACAUUGAAAAUU-CAGCAAU 3') was chemically synthesized by an external company (Metabion, Planegg, Germany). For other target RNAs, the DNA templates for the in vitro transcription were obtained by DreamTaq DNA polymerase (Thermo Fischer Scientific, Waltham, Massachusetts, USA)

extension of overlapping oligodeoxyribonucleotides (GENOMED S.A., Warsaw, Poland; Supplementary Table S7), as previously described [65].

RNA transcription with T7 RNA polymerase was conducted according to the procedure presented by [66]. Then, RNA molecules were purified using denaturing gel electrophoresis and 5' ³²P-labeled (HARTMANN ANALYTIC GmbH, Braunschweig, Germany) with T4 polynucleotide kinase (Thermo Fischer Scientific, Waltham, Massachusetts, USA). Obtained samples were treated with the phenol-chloroform mixture (BioShop, Burlington, ON, Canada) and purified on P-30 spin columns (Bio-Rad Laboratories, Hercules, California, USA). Finally, ³²P-labeled RNAs were precipitated by ethanol (Avantor Performance Materials Poland S.A., Gliwice, Poland) and used for binding assays.

Protein- and RNA-binding assays were conducted according to a previously described procedure [66]. Briefly, ³²P-RNA molecules were denatured at 90 °C for 2 min and refolded during incubation on ice for 5 min. The different concentrations of unlabeled RNAs or proteins were obtained by their serial dilutions prepared in the binding buffer, which contained: 24 mM Tris-HCl (pH 7.5; VWR International, Radnor, Pennsylvania, USA), 150 mM NaCl (Chempur, Piekary Slaskie, Poland), 5% glycerol (STANLAB Sp. z o.o., Lublin, Poland), 100 µg/ml BSA (Thermo Fischer Scientific, Waltham, Massachusetts, USA), and 1 mM MgCl₂ (Merck KGaA, Darmstadt, Germany). Then, diluted RNAs or proteins were mixed in a 1:1 ratio with 2 nM ³²P-RNAs in the binding buffer (see above). Incubations of mixtures for RNA-RNA assays were conducted for 4 h (in case of 5'UTR *roi* mRNA – UpRoi1 pair) or 3 h (in case of *flgL* mRNA – UpRoi1 pair) at 4 °C or room temperature, while for RNA-Hfq or RNA-ProQ assays were performed for 30 min at room temperature or 4 °C, respectively. In the next step, 5 µl of each mixture (or 7 µl in case of *flgL* mRNA – UpRoi1 pair) were loaded onto a native 6% polyacrylamide gel (19 acrylamide:1 bis-acrylamide; Merck KGaA, Darmstadt, Germany), and run in 0.5× or 1× TBE (Thermo Fischer Scientific, Waltham, Massachusetts, USA). The radioactive gels were visualized using a phosphorimager (Typhoon 9200, GE Healthcare). The equilibrium dissociation constant (*K*_d) values were estimated by using the GraphPad Prism software and the quadratic equation as presented earlier [65].

4.10. Overproduction and purification of the *E. coli* Hfq and ProQ proteins

The Hfq and ProQ proteins were purified as described earlier [66,67], respectively.

4.11. Gel mobility shift assay for detecting protein-nucleic acid interactions

To detect LexA protein complexes with a 280 bp fragment harboring the promoter *pN* region located upstream of UpRoi1 nucleotide sequence in *E. coli* MC1061 (Φ 24_B) genome, the gel electrophoresis mobility shift assay (EMSA) was employed [68]. Briefly, the genomic DNA of *E. coli* MC1061 lysogenic with bacteriophage Φ 24_B as a template for PCR reaction was isolated by using the NucleoSpin® Tissue kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany), following the manual of manufacturer. The region encompassing the nucleotide sequence of *pN* promoter was amplified in the PCR reaction with two specific oligonucleotides: F_pN (5' GAATTCGGCGACAAGTCTAG 3'; GENOMED S.A., Warsaw, Poland) and R_pN (5' AAAGCGTGGGGT-GATTCAAC 3'; GENOMED S.A., Warsaw, Poland). The obtained DNA fragment was purified from the agarose gel using the GeneMATRIX Agarose-Out DNA Purification Kit (EURx Sp. z o.o., Gdansk, Poland). T4 polynucleotide kinase (Thermo Fischer Scientific, Waltham, Massachusetts, USA) was employed to end-label the purified fragment of DNA with [γ -³²P] ATP (HARTMANN ANALYTIC GmbH, Braunschweig, Germany), according to the instructions from the provider. The non-incorporated [γ -³²P] ATP was removed from the DNA sample using

the Micro Bio-Spin™ 30 Columns, RNase-Free (Bio-Rad Laboratories, Hercules, California, USA). Then, 0.1 fmol of the labeled DNA fragment was incubated with various concentrations of LexA protein ([Antibodies.com](https://www.antibodies.com) Europe AB, Stockholm, Sweden) for 30 min at 30 °C in 20 µl of reaction mixture containing 1× binding buffer: 10 mM Tris-HCl (pH 8; VWR International, Radnor, Pennsylvania, USA), 50 mM NaCl (Thermo Fischer Scientific, Waltham, Massachusetts, USA), 5 mM MgCl₂ (Merck KGaA, Darmstadt, Germany), 1 mM EDTA (Thermo Fischer Scientific, Waltham, Massachusetts, USA), 5% glycerol (STANLAB Sp. z o.o., Lublin, Poland), 1 mM DTT (VWR International, Radnor, Pennsylvania, USA) and 0.01 mg/ml BSA (Thermo Fischer Scientific, Waltham, Massachusetts, USA). After incubation, the reaction mixtures were resolved by 7% native PAGE in 1× TBE buffer (Thermo Fischer Scientific, Waltham, Massachusetts, USA). The radioactive gels were visualized using a phosphorimager (Typhoon 9200, GE Healthcare).

4.12. Transcriptomic analysis using RNA-seq

For RNA-seq, *E. coli* MC1061 (Φ24_B) host cells bearing plasmid pBAD24, or pBAD24_pN_UpRoi1 were cultivated in LB medium at 37 °C to an OD₆₀₀ of 0.1. Then, bacterial cultures were diluted to an OD₆₀₀ of 0.001 in LB medium. Following the prophage induction with 1 µg/ml of mitomycin C (BioShop, Burlington, ON, Canada), bacteria were grown at 37 °C with shaking. Total RNA was isolated from 7.5×10^8 bacterial cells harvested at 210 min after prophage induction using the RNeasy Mini Kit (Qiagen, Hilden, Germany). To remove bacterial DNA from the samples, the TURBO DNA-free™ Kit (Thermo Fischer Scientific, Waltham, Massachusetts, USA) was employed. Following enzymatic reactions, RNAs were cleaned up and concentrated with the RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany), according to the procedure described in the manual. The integrity and quantity of RNA samples were assessed using the RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA) of the Agilent 2100 Bioanalyzer System (Agilent Technologies, Santa Clara, CA, USA). Isolation of ribonucleic acids from bacterial cells was performed from 4 independent biological repeats.

The mRNA libraries, sequencing, and data analysis were prepared by the Novogene company (Novogene (UK) Company Limited, Cambridge, United Kingdom). Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (New England Biolabs, Ipswich, Massachusetts, USA) following the manufacturer's recommendations, and index codes were added to attribute sequences to each sample. Briefly, mRNAs were purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5×). The first strand of cDNA was synthesized using a random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-). Second-strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of the 3' ends of cDNA fragments, the NEBNext Adaptor with hairpin loop structure was ligated to prepare for hybridization. To select cDNA fragments of preferentially 150–200 bp in length, the library fragments were purified with the AMPure XP system (Beckman Coulter, Beverly, USA). Then 3 µl USER Enzyme (New England Biolabs, Ipswich, Massachusetts, USA) was used with size-selected, adaptor-ligated cDNA at 37 °C for 15 min followed by 5 min at 95 °C before PCR. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers, and Index (X) Primer. At last, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system.

The clustering of the index-coded samples was performed on a cBot Cluster Generation System using PE Cluster Kit cBot-HS (Illumina, Inc., San Diego, California, USA) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina platform, and paired-end reads were

generated.

4.13. Analysis of RNA sequencing data

Raw reads of FASTQ format were firstly processed through fastp. In this step, clean data (clean reads) were obtained by removing reads containing adapter, reads containing ploy-N and low-quality reads from raw data. At the same time, Q20, Q30, and GC content for the clean data were calculated. All the downstream analyses were based on clean data with high quality. Reference genomes (*E. coli* no. U00096.3 and Stx phage no. NC_027984.1) and gene model annotation files were downloaded from the genome website directly. The index of the reference genome was built using Bowtie2 and paired-end clean reads were aligned to the reference genome [69]. FeatureCounts was used to count the reads numbers mapped to each gene. Fragments per kilobase per million mapped fragments (FPKM) of each gene were calculated based on the length of the gene and the reads count mapped to this gene [70]. Differential expression analysis of two groups was performed using the DESeq2 R package [71]. Gene Ontology (GO; <https://geneontology.org/>) was used to annotate genes to biological processes [72,73]. Kyoto Encyclopedia of Genes and Genomes (KEGG; <https://www.genome.jp/kegg/>) was employed to annotate genes to pathway level [74].

4.14. Gene expression analysis by RT-qPCR

For RNA isolation, bacterial samples were prepared according to the procedure described in the above section. Total RNA was extracted from 1×10^9 bacterial cells harvested at 190 and 210 min after prophage induction with 1 µg/ml of mitomycin C (BioShop, Burlington, ON, Canada) by using the High Pure RNA Isolation Kit (Roche Diagnostics International, Rotkreuz, Switzerland). To remove DNA contamination from RNA samples, the TURBO DNA-free™ Kit (Thermo Fischer Scientific, Waltham, Massachusetts, USA) was employed. The RNA concentration and quality were analyzed by a NanoDrop OneC spectrophotometer (Thermo Fischer Scientific, Waltham, Massachusetts, USA). Preparation of cDNA from 1.25 µg of RNA samples for RT-qPCR was performed with Transcriptor Reverse Transcriptase (Roche Diagnostics International, Rotkreuz, Switzerland) and random hexamer primers (Roche Diagnostics International, Rotkreuz, Switzerland), according to the manufacturer's instructions. Finally, mixtures were 10-fold diluted in DEPC-treated water (Thermo Fischer Scientific, Waltham, Massachusetts, USA) and tested in RT-qPCR. The pattern of gene expression after prophage induction was determined by using the LightCycler® 480 Real-Time PCR System (Roche Diagnostics International, Rotkreuz, Switzerland) and LightCycler® 480 SYBR Green I Master Mix (Roche Diagnostics International, Rotkreuz, Switzerland) as described previously [14,15,64]. Transcription rates of tested genes were compared in parallel to the *icdA* housekeeping gene [62]. All DNA oligonucleotides (Supplementary Table S7) were designed by Primer3-web (version 4.1.0) and synthesized by GENOMED S.A. (Warsaw, Poland). Each reaction mixture consisted of 2× SYBR Green I Master Mix, 6.25 ng/µl cDNA, and 200 nM primers. Real-time PCR amplification was performed with the following program: 95 °C for 5 min, 55 cycles of 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 15 s. A melting curve analysis confirmed the specificity of the designed DNA oligonucleotides. The ratio *R* of tested genes expressed in *E. coli* MC1061 (Φ24_B) [pBAD24_pN_UpRoi1] vs. that expressed in the control variant *E. coli* MC1061 (Φ24_B) [pBAD24] was calculated by using the Pfaffl method [75]. PCR efficiency was determined for each tested gene by the Lin-RegPCR program [14,15,64]. All data of gene expression are the average of triplicate analyses.

4.15. Proteomic analysis based on LC-MS/MS data

4.15.1. Protein sample preparation

Samples for proteomic analyses were prepared according to the

procedure described earlier [76], with slight modifications. Briefly, *E. coli* MC1061 (Φ 24_B) host cells bearing plasmid pBAD24 or pBAD24_pN_UpRoi1 were cultivated in liquid LB medium at 37 °C to an OD₆₀₀ of 0.1. Then, bacterial cultures were diluted 100-fold in fresh LB medium. The growth of the tested variants was continued until 210 min in the presence of mitomycin C (BioShop, Burlington, ON, Canada) at a final concentration of 1 µg/ml. In the next step, 2×10^9 bacterial cells from each sample were collected, centrifuged (7000 ×g, 5 min, 4 °C), and resuspended in a lysis buffer (1.5% SDS, 0.1 M DTT, 0.1 M Tris-HCl pH 7.6). The mixtures were incubated for 7 min at 95 °C and then kept at –20 °C until further mass spectrometry measurements.

4.15.2. Protein digestion and mass spectrometry analysis

A bottom-up proteomics approach was employed, where protein digestion was performed for each sample using the Filter-Aided Sample Preparation (FASP) method with 10 kDa molecular weight cut-off filters (Microcon, Merck Millipore) [77]. 100 µg of protein was used for digestion, with reduction carried out directly in the lysis buffer by the addition of dithiothreitol (DTT), followed by alkylation with iodoacetamide (IAA). Enzymatic digestion was conducted overnight at 37 °C using trypsin.

Peptide purification was carried out using C18 StageTips [78]. After evaporation, the peptide samples were resuspended in 30 µl of water containing 1% acetic acid and analyzed using a TripleTOF 5600+ mass spectrometer (Sciex, Framingham, MA, United States) coupled with an Eksper MicroLC 200 Plus System (Eksigent, Redwood City, CA, United States) equipped with a ChromXP C18CL column (3 µm, 120 Å, 150 × 0.3 mm).

Peptide separation was performed using a gradient elution of 11–42.5% solvent B over 60 min, where solvent A was 0.1% formic acid in water, and solvent B was 0.1% formic acid in acetonitrile. Instrumental parameters were applied as previously described [79].

Label-free relative quantitative analysis was conducted using the SWATH-MS (Sequential Window Acquisition of All Theoretical Mass Spectra) approach, with variable isolation windows generated via SwathTUNER. A uniform distribution of precursor ions with charges ranging from +2 to +5 was considered. The total acquisition cycle lasted 1.1 s, with an ion accumulation time of 50 ms per window. Product ion scanning was performed across a 100–1800 *m/z* range, with an acquisition time of 40 milliseconds per window. The spectral library required for SWATH-MS analysis was constructed by processing all spectra acquired in data-dependent acquisition (DDA) mode using PeakView v2.2 (Sciex). Protein identification was performed using ProteinPilot 4.5 (Sciex), employing the Paragon algorithm, with database searches conducted against UniProt for *E. coli* (downloaded in April 2023). The false discovery rate (FDR) was controlled at 1%. For data-independent acquisition (DIA) analysis, spectra from each sample were compared against the previously generated spectral library using PeakView. The resulting quantitative data were exported to MarkerView, normalized using the log2 transformation, and subjected to statistical analysis in Perseus. The mass spectrometry proteomics dataset has been deposited in the ProteomeXchange Consortium via the PRIDE repository [80]. Logging in to the PRIDE website is possible using the details described in Section 4.20. Finally, STRING 11.0 was utilized to construct protein interactome networks and conduct Gene Ontology (GO) enrichment and KEGG pathway analysis for differentially expressed proteins.

4.16. Cell viability analysis using flow cytometry

E. coli MC1061 cells lysogenic for Φ 24_B bacteriophage and bearing plasmid pBAD24, pBAD24_UpRoi1, or pBAD24_pN_UpRoi1 were cultured in LB medium at 37 °C to an OD₆₀₀ of 0.1. Then, bacterial cultures were diluted to an OD₆₀₀ of 0.001 in a fresh LB medium and treated with 1 µg/ml of mitomycin C (BioShop, Burlington, ON, Canada). At 300 min after induction, samples were withdrawn and centrifuged (10,000 ×g, 3 min, room temperature). The supernatants were

removed, and the obtained pellets were washed in 0.85% NaCl (Chempur, Piekary Slaskie, Poland). Bacterial suspensions were stained with LIVE/DEAD BacLight™ Bacterial Viability and Counting Kit (Thermo Fischer Scientific, Waltham, MA, USA), according to the manufacturer's protocols. The mentioned kit consisted of two types of fluorescent nucleic acid stains: SYTO9 (excitation/emission maxima = 480/500; FITC-A collecting SYTO 9) and propidium iodide (PI; excitation/emission maxima = 490/635; PerCP-Cy5.5-A collecting PI), which labeled live and dead bacteria, respectively. The green fluorescent dye SYTO9 stained bacterial cells with both intact and damaged membranes. In contrast, the red-fluorescence dye PI penetrated only into bacterial cells with damaged membranes, causing a reduction in the green fluorescence signal when both stains were used [81]. The BD FACSLytic™ Clinical Cell Analyzer (BD Biosciences, San Jose, CA, USA) was employed to enumerate and visualize live/dead bacteria. The BD FACSuite™ Software was used to analyze the data, create a dividing line based on a dead-only control (ethanol-treated cells), distinguish between dead/live bacterial cells, and perform percentage evaluations.

4.17. Materials for UV spectrophotometric measurements and CD spectroscopy

RNA Uproi1 molecule was purchased from Metabion International AG (Germany) and prepared as 10 µl matrix solution with the concentration established at 466.1 ng/µl. The 100-nt long fragment of 5'-UTR *roi* mRNA and 85-nt long fragment of *flgL* mRNA were obtained by transcription in vitro (described in Section 4.9) at a concentration equal to 1000 ng/µl. RNAs' solutions were prepared through matrix desolvation in 1.5 ml biological buffer (regarding the composition: 24 mM Tris, 140 mM NaCl, 1 mM MgCl₂, 5% glycerol, pH 7.5) and ensured the formation of a homogeneous solution.

4.17.1. UV spectrophotometric measurements

Electronic spectra were recorded on an Evolution 300 spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA) in the range of 200–400 nm for UV detection of intermolecular interactions with biomolecules, together with a spectral band with a width of 2 nm. The computer-controlled automatic microtitrator CerkoLab, equipped with a Hamilton syringe (0.5 ml or 1.0 ml), was used to introduce a precise volume of 0.00416 ml of 5'-UTR *roi* mRNA or 0.0083 ml of *flgL* mRNA in each measurement step.

Spectrophotometric analysis confirmed initial concentrations of 0.031 µM for 5'-UTR *roi* mRNA and 0.025 µM for *flgL* mRNA, with the corresponding titrant UpRoi1 at concentrations of 0.73 µM and 0.33 µM, respectively. The binding ability was investigated using spectrophotometric titrations. The UpRoi1 molecule played the role of titrand, and its 0.5 ml solution was placed in a quartz cuvette. The 5'-UTR *roi* mRNA and *flgL* mRNA solutions were used as titrants. A time interval of 120 s was maintained between successive titrant additions, comprising a 90-second equilibration phase followed by a 30-second mixing period. A total of 90 titrant additions were performed under controlled conditions. The initial experimental conditions were systematically established and the corresponding electronic absorption spectra of the precursor solutions are shown in Supplementary Fig. S4. Additionally, all original registered as spectra-sets recorded along with the dilution effect have been presented in Supplementary Fig. S5.

4.17.2. Thermodynamic and binding parameters determination

Importantly, the preliminary step of results elaboration involved eliminating the dilution effect. Based upon the variation in absorbance, the association/binding constants K_{bind} of these interactions with UpRoi1 were determined according to the Benesi–Hildebrand Eq. (1) presented below [82]:

$$\frac{A_0}{A - A_0} = \frac{\epsilon_G}{\epsilon_{H-G} - \epsilon_G} + \frac{\epsilon_G}{\epsilon_{H-G} - \epsilon_G} \cdot \frac{1}{K_{bind}[\text{mRNA}]} \quad (1)$$

where:

A_0 and A are the absorbances of the UpRoi1 derivative in the absence and presence of tested mRNA, respectively; ϵ_G and ϵ_{H-G} are the absorption coefficients of the mRNA unbound and its complex with UpRoi1, respectively. Thus, the double reciprocal plot of $A_0/(A - A_0)$ versus $1/[mRNA]$ is linear, and the binding constant (K_{bind}) was estimated based on the linear fit parameters established as the ratio of the intercept to the slope [83,84]. The binding data were fitted using Origin2022b software (OriginLab Corporation, Northampton, MA, USA).

Based on the Eq. (2) and known values of binding constants K_{bind} the free Gibbs energy of all compound interactions were determined.

$$\Delta G = -R \cdot T \cdot \ln(K_{bind}) \quad (2)$$

where: ΔG - free Gibbs Energy; R - molar gas constant [$J/mol \cdot K$]; T - temperature [K]; K_{bind} - the value of binding constant (formation of the complex).

4.17.3. CD spectroscopy

CD spectra were recorded at pH 7.5 with a Jasco J-815 spectropolarimeter. For measurements in the Far-UV region (190–320 nm), a quartz cell with a path length of 0.1 cm was used. Three scans were accumulated at a scan speed of 50 nm per minute, with data being collected at every 0.2 nm from 200 to 320 nm. CD measurements were performed in parallel with UV absorption measurements. The absorbance band of the UpRoi1-5'-UTR *roi* mRNA or UpRoi1- *flgL* mRNA complexes was generated based on the UV spectra obtained in the last step of spectrophotometric titration. Spectra were corrected for buffer signal and converted to the $\Delta\epsilon$ in [$mol \cdot dm^{-3} \cdot cm^{-1}$] according to the relationship:

$$\Delta\epsilon = \frac{\theta}{32,980 \cdot C \cdot L \cdot N} \quad (3)$$

where:

θ is the measured (raw) CD amplitude in [mdeg]; C is the sample concentration in [$mol \cdot dm^{-3}$].

L is the cell path length $L = 0,1$ [cm]; N is the number of nucleotides of the RNA.

4.18. Bacterial motility test

Detection of bacterial motility on semi-solid agar was conducted as described previously [85], with some modifications. Briefly, *E. coli* MC1061 ($\Phi 24_B$) host cells bearing plasmid pBAD24, pBAD24-UpRoi1, or pBAD24_pN-UpRoi1 were grown in LB medium at 37 °C to an OD_{600} of 0.1. Then, bacterial cultures were diluted 100 times in LB medium, and 60 min after dilution, 1.46×10^7 bacterial cells were withdrawn and injected into a semi-solid LB medium (0.3% agar) plate. After 18 h incubation at 37 °C, photographs of the Petri dishes were taken (UVITEC Alliance Q9), and the diameters of the opaque areas were measured.

4.19. In silico analyses

Prediction of putative promoters in the nucleotide sequence of the genome of $\Phi 24_B$ bacteriophage (NC_027984.1) [55,86] was performed using BPROM [87]. Promoters were searched within a 1475 bp long sequence fragment (from base 18540 to 20014) encompassing the *ant* and *roi* genes. Putative Rho-independent terminators were identified using ARNold, which employs RNAmotif and ERPIN complementary programs [88–90]. In turn, Rho-dependent terminators were predicted by RhoTermPredict software [91]. Secondary structures of tested RNA fragments were generated using the RNAstructure program [92]. IntaRNA was employed to predict interactions between two RNA molecules [93–96]. The identification of the potential binding sites for UpRoi1 sRNA was based on a novel algorithm that is presented in this

work for the first time and described in detail in Supplementary Table S1. This original algorithm was developed based on available knowledge [97–105]. The determined binding sites were next identified in individual genes of the *E. coli* genome using Clone Manager software.

4.20. Statistical analysis

All experiments were carried out in 3 or 4 biological replicates. The means $\pm$ SD were used in the statistical analysis of the data and the graphics. A comparison of two averages in physiological and proteomic experiments was performed using Student's *t*-test. The significant differences were marked by asterisks as follows: $p < 0.05$ (*), $p < 0.01$ (**), or $p < 0.001$ (***). In the transcriptomic analyses, DESeq2 was used to provide statistical routines for determining differential expression in digital gene expression data based on the model of the negative binomial distribution. The resulting *p*-values were adjusted using Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with an adjusted *p*-value < 0.05 found by DESeq were assigned as differentially expressed. GO enrichment analysis of differentially expressed genes was implemented by the clusterProfiler R package, in which gene length bias was corrected [106]. GO terms with corrected *p*-value less than 0.05 were considered significantly enriched by differentially expressed genes. The clusterProfiler R package was also employed to test the statistical enrichment of differential expression genes in KEGG [107].

CRedit authorship contribution statement

Sylwia Bloch: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Natalia Lewandowska:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Joanna Zwolenkiewicz-Nowak:** Visualization, Validation, Methodology, Investigation, Data curation. **Monika Mazur:** Visualization, Validation, Methodology, Investigation, Data curation. **Agnieszka Chylewska:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Aleksandra M. Dąbrowska:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Natalia Musiał:** Writing – review & editing, Methodology, Investigation. **Paulina Czaplewska:** Writing – review & editing, Methodology, Investigation. **Wojciech Wesolowski:** Investigation, Data curation. **Aleksandra Łukasiak:** Data curation. **Mariusz Ferenc:** Writing – review & editing, Methodology. **Marcin Pietrzycki:** Methodology. **Emilia Węglińska:** Data curation. **Paulina Mach:** Investigation, Data curation. **Grzegorz Węgrzyn:** Writing – review & editing, Formal analysis. **Mikołaj Olejniczak:** Writing – review & editing, Supervision, Methodology, Formal analysis, Conceptualization. **Bożena Nejman-Faleńczyk:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbagr.2026.195135>.

Data availability

Data are available via ProteomeXchange with the identifier PXD062220. Until published data is private and unavailable to every user. For verification and review purposes, a login (reviewer_pxd062220@ebi.ac.uk) and password (ChDGBErHLVMG) have been generated to allow viewing of the deposited data. Alternatively, logging to the PRIDE website is also possible using the project accession no: PXD062220 and token: zeCFhh0tIPFR.

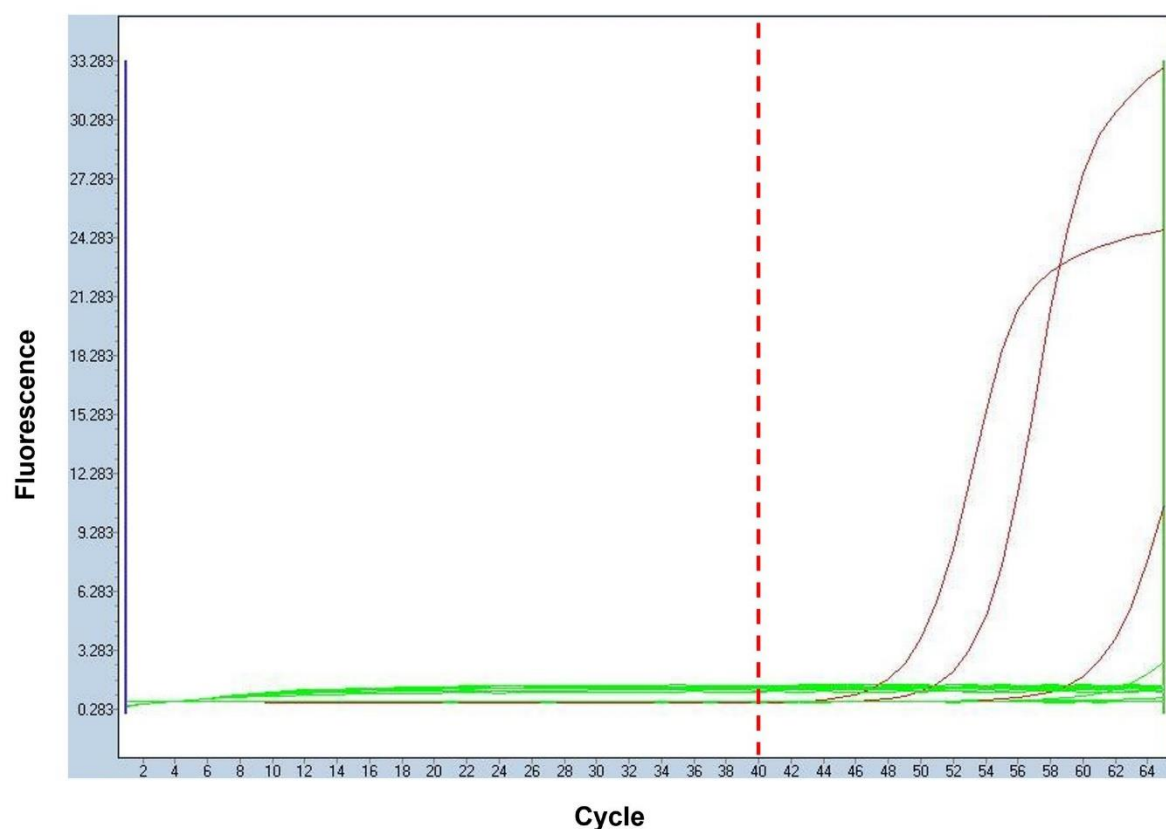
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Suplement do pracy nr 3



Supplementary Fig. S1. Fluorescence vs. cycle number plot of RT-qPCR results showing the lack of UpRoi1 product in non-lysogen strains *E. coli* [pBAD24], *E. coli* [pBAD24_UpRoi1], and *E. coli* [pBAD24_pN_UpRoi1] as no fluorescence signal was observed below the 40th cycle, which is marked with a red dotted line. A sporadic fluorescence signal appearing above the 40th cycle is commonly interpreted as a result of probe contamination and does not indicate the formation of a target product [106].

Supplementary Fig. S2. Panel A

A

rut site of T3_Rho terminator

1 atggctgttc aaatttctgt cgaaaacctt tccctgtta cctataacca gatecccgta
 61 attactactg aactgttggc tcacctttac qgaacaaaa tgaaaaacat ttctgataac
 121 ttctgaaca acacgacgcg attcatgccc ggaagcatt actttaaaat tgaaaaaac
 181 gaattacgcg agtttaagaa cagacccgaa acaatcggt tagttgtaa aaatgccgc
 241 tccctcatcc tctggacaga acgagcgca gcccgtcacg caaaaatgct cgaaactgat
 301 caggcgtggg atgtgttga aaaactggaa gactgctatt tcagccagtgc cgagaaaaat

rut site of T1_Rho terminator

361 actggcaaac aagagaagaa gctcaacggg ctttccgcaa aagaacaga cagccttqta
 421 tggtgtggg attatgcaa ccgctcacag gcattgttc gtgagttgta tcccgatta
 481 aaactgattc agtctggcta ttccggcata tgccacgact acggctataa gttctcgat
 541 atcatcgga gggcgagggg cgttttaatt aatcacacgc gggatataga tatttatgag
 601 cctgacggc cgacgaacct tctggcatgg gaaaggctta agaacaaga gttgccgcct

p946 -35 box p946 -10 box

661 tcactgcato gctactgaca attgacaact taacaaaccc agcttcggct gggtttttt

T1 terminator

721 tgcctgaatt ttcaatgtga gaggacatga caatgaatga gctgataaat agcaatgcca

781 tcaaaatgac aagcattgaa atcgctgagt tgggtggaag ccaacacggt aatgtcagaa
 841 tatcaataga acgtctggca aagcgtggg tgattcaact tccttcaatg caaaaagt
 901 aaaataaaca acaattagc ctaacaaat tcacaagcgt gtatatattc gaaggcgaac
 961 aaggtaagcg agacagcatt attgtcgtc ctcagttgtc gccggaatto accgcteqcc

rut site of T2_Rho terminator

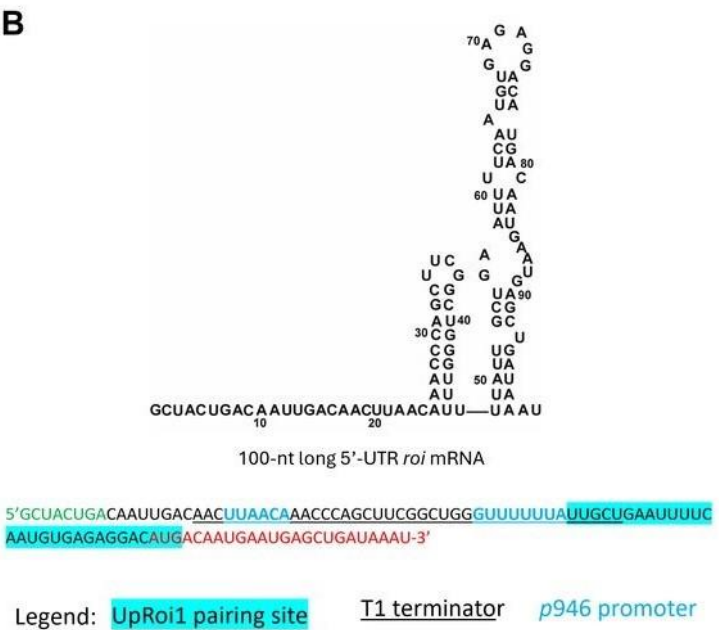
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 1081 aggcattgcg ccttgcggcc gacctgaag accagaaggc tgaactggag aaacagcttg
 1141 ctctcgacgc acctaaagt gagtttgccg atcgcttg cgaggccagc ggaattttga
 1201 ttgaaaactt tgcaaagggt gttgtattg gtccaaacaa actgtttgcg tggatgcgcg
 1261 atcacaaaat ccttattgct tcaggttccc ggcgaaatgt gccaatgcag gaatatatg
 1321 agcgcgggta tttcacagt aaagaaacag cggtaatac aatcacgga atacagatat
 1381 cgttcaccac aaaaatcacc gggcgtggc aacagtggc gacaagaaag ctgctagata
 1441 acggaatgct taaagtaaca ggggaggctg cttaa

Legend:

UpRoi1 pairing site T1 terminator p946 promoter 100-nt fragment

The Rho binding sites (*rut*) of Rho-dependent transcription terminators T1_Rho, T2_Rho and T3_Rho, are underlined and marked in bold

Supplementary Fig. S2. Panel B



Supplementary Fig. S2. Panel C

C

```
>HM208303.1:c20014-18322 Stx2 converting phage vB_EcoP_24B, complete
genome

TTAAGCAGCCTCCCCTGTTACTTTAAGCATTCCGTTATCTAGCAGCTTCTTGTGAGCCACTGTTGACCA
CGCCCGGTGATTTTGTGGTGAACGATATCTGTATTCCGTGATTTGTATTGACCGCTGTTTCTTTCACTG
TGAAATAGCCGCTCCATATATTCCTGCATTGGCACATTCGCCGGGAACCTGAAGCAATAAGGATTTT
GTGATCGCGCATCCACGCAACAGTTTGTGTTGGACCAATACCAACAACCTTTGCAAAGTTTCCAATCAAA
ATTCCGCTGGCCTCGCCAACGCGATCGGCAAACTCAACTTTAGGTGCTGCGAGAGCAAGCTGTTTCTCCA
GTTGAGCCTTCTGGTCTTCAAGTTCGCCGCAAGGCGCAATGCCTCAGAAAAGGTTTGTGGTATTTTCGC
GGTTGCCCTTCGAGTTCTCGCCAGCGGTCAACAAGCGAGCGGTGAATTCGCCGACAACTGAGCGACG
                                LexA binding sites      pN -35 box

ACAATAATGCTGTCTCGCTTACCTTGTTTCGCCTTCGAATATATACGCTTGTGAATTTGTTAGGGCTAA
                                pN -10 box

TTGTTTGTATTATTTCAACTTTTGCATTGAAGGAAGTTGAATCACCCACGCTTTGCCAGACGTTCTAT
TGATATTCTGACATTACCGTGTGGCTTCCACCAACTCAGCGATTTCATGCTTGTCAATTTGATGGCA
                                UpRoil sequence      T2 terminator
TTGCTATTATCAGCTCATTCAATGTCATGTCCTCTCACATTGAAAATTCAGCAATAAAAAACCCAGCCG
                                ←

AAGCTGGGTTTGTTAAGTTGTCGAATTGTCAGTAGCGATGCAGTGAAGCGGCAACTCTTTGTTCTTAAGC

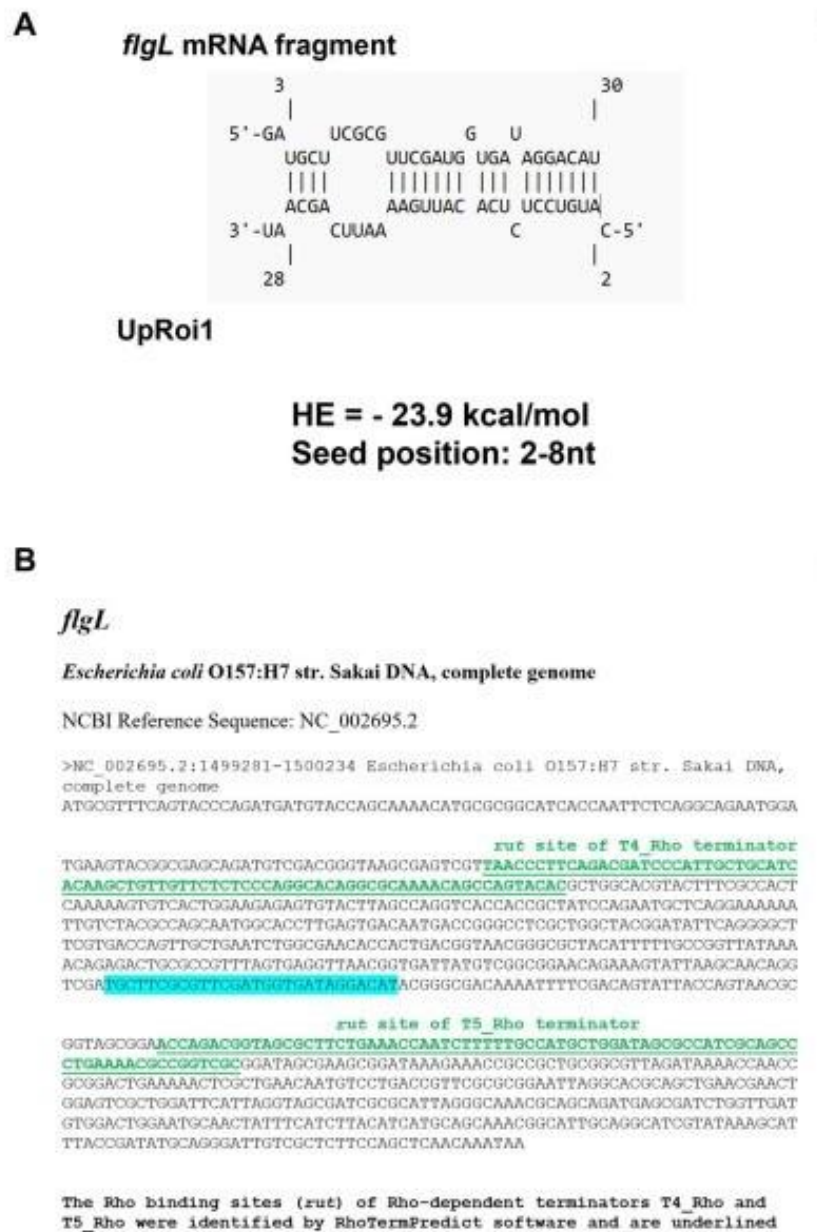
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TTTTGCGTGACGGGCTGCGCCTCGTTCGTCCAGAGGATGAGGGAGCGGGCATTTTTACCAACTAACCCG
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TGAATCGCGTCTGTTGTTTCAGAAAGTTATCAGAAATGTTTTGATTTTGTTCGGTAAAGGTGAGCCAA
CAGTTCAAGTAGTAATTACGGGGATCTGGTTATAGGTAACAGGGGAAAGGTTTTCGACAGAAATTTGAACA
GCCATAATGACCTCGCGTTTCGATAATTTTACCTCGCCACCGTCAGGTGCTAATCATCGTGGTGGCGAA
CTGTGCGGGGTTAGCACTACCGGTCGAAACATCCGGCGAGCCTTTCGGCTCCCCACACAGCCGCCATA
AATCGCGAATGTGACTGTGCTTAGCGCATAAAAAACCGCCAGCGCGGTTATGCACCGTTTCGATATCCGG
GGTGCTAATCCCG
```

Legend:

ATGTCCTCTCACATTGAAAATTCAGCAAT UpRoil sequence
 GTTTATTTT - 10 box of pN promoter
 TTGTTA - 35 box of pN promoter
 ATATATA LexA two binding sites that overlap
 AGCAATAAAAAACCCAGCCGAAGCTGGGTTTGTTAAGTTGTC - T2 transcription terminator
 sequence
 The sequence complementary to roi coding sequence is marked in red. The
 arrow indicates the position of ATG start codon located on the opposite
 strand.

Supplementary Fig. S2. Fragments of $\Phi 24_B$ phage genome sequence encompassing the *roi*, *ant*, and *uproi1* genes. (A) Fragment of phage genome encompassing *ant* (green) and *roi* (red) sequences with indicated pairing site (blue) for UpRoi1 within 5' UTR of the *roi* gene. The purple box indicates the 100 nt-long transcript fragment used for further binding analysis. The *p946* promoter boxes -35 (TTAACA) and -10 (GTTTTTAT) of the *roi* gene are marked in blue, and the T1 terminator (Rho-independent) located between the *ant* and *roi* genes is underlined (AACTTAACAAACCCAGCTTCGGCTGGGTTTTTATTGCT). Importantly, the sequences of the *p946* promoter and T1 terminator overlap. Downstream and upstream of them, there are located additional Rho-dependent terminators, T1_Rho, T2_Rho, and T3_Rho, whose *rut* sites, for binding of Rho protein, are underlined and marked in bold. (B) Sequence and secondary structure of 100-nt long RNA fragment containing the region between the *ant* and *roi* coding sequences. The sRNA secondary structure was visualized by the RNAstructure program (<https://rna.urmc.rochester.edu/RNAstructureWeb/>) and presented schematically. (C) Fragment of phage genome encompassing *uproi1* gene with highlighted sequences of *pN* promoter -35 (TTGTTA) and -10 (GTTTATTTT) boxes, T2 terminator (AGCAATAAAAAACCCAGCCGAAGCTGGGTTTGTTAAGTTGTC), and LexA binding sites TATATACA and ATATATAC that overlap.

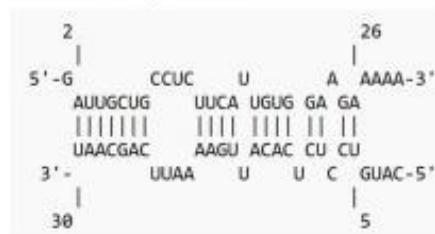
Supplementary Fig. S3. Panels A and B



Supplementary Fig. S3. Panels C and D

C

oppA mRNA fragment



UpRoi1

HE = - 18.5 kcal/mol
Seed position: 24-30nt

D

oppA

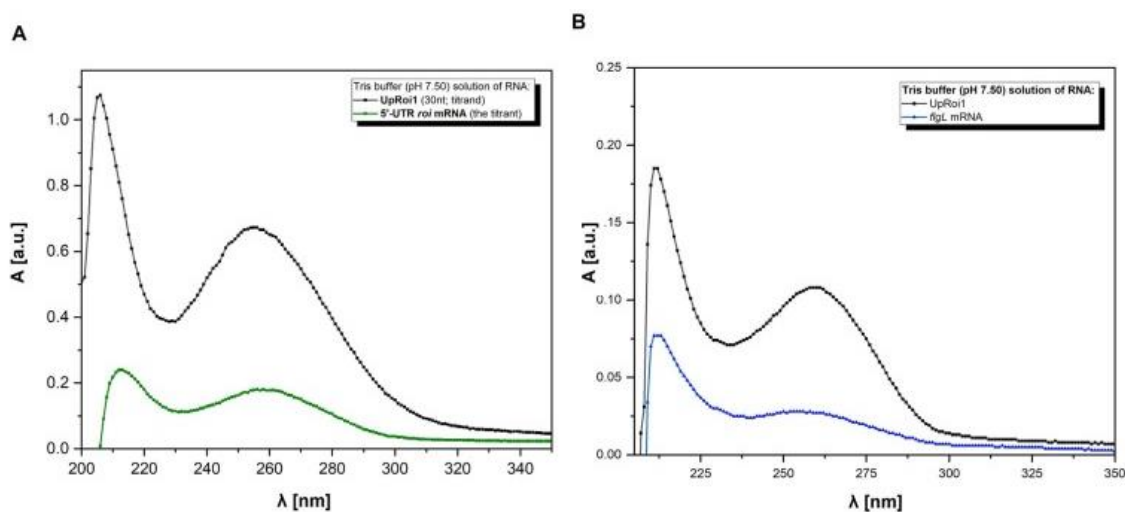
Escherichia coli O157:H7 str. Sakai DNA, complete genome

NCBI Reference Sequence: NC_002695.2

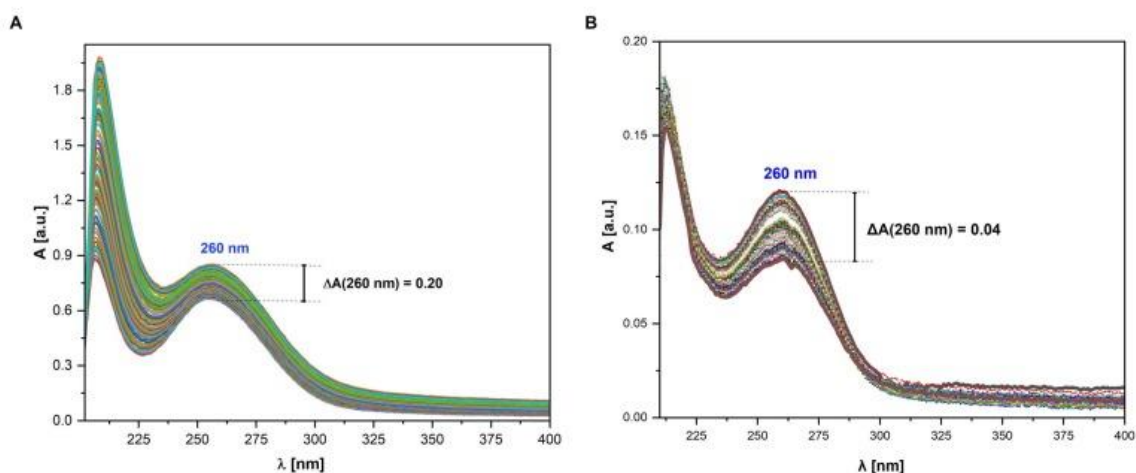
5'NC 002695.2:1744118-1745794 Escherichia coli O157:H7 str. Sakai DNA, complete genome

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TAGTAGCAGCTGCGCTTCTGGCTCGGCTAATGGCAGGGAAATGTCGGCTGGCAGCTGATGTACCCGACGG
CGTCACACTGGCGGAAAAACAACGCTGGTACGTAACAATGGTTCAGAAGTTCAGTCATTAGATCCGCAG
AAAATTGAAAGGTTTCCGGAGCTTAATATCAGCGACGACCTGTTTGAAGGCTTACTGCTCAGCAGATCTTG
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GCGTAAAGATGCGAAATGGTCTGATGGCAGCGCAGTCACAGCACAAAGACTTTGTGTATAGCTGGCAACGT
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TAAAAAGATTGGGTCGTAACACGAAATCGTTCTTGAACGACGCCAACCTACTGGAAACACCGGAAAT
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GAATGACATGATTTATTAACAACATGCGCGATCGAATTGTTCTCGAAGAGCTGAAAAAGAGATCCGGACG
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TGTGCGTGTGCGTACCGCATGAACCTGGGATGAGCAGCGATATCTGTTTAATAAAGTGAAGCGCGAG
GGCCAGATTCGCCCGGATAATGTTTACACTCCACGCTATCTGATGGCGCAAAATTGACTCAGCGCGAATGGT
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CAACCGCTTGACCATCAACCTGTGTGATAACACCTCCGATTCGATAAAGAACTGGCCATTTGCGCGGCT
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CCGCTCACCAGGGGTACTTTTGTGTGGCCCGGTGACGGGCTGGTGTGCTGACTACCAACGAAACCTCTTCCT
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TGGATAAGGATTCCGGCATTTGCTCTGTTTATTACTACGTAAGTGGCGCTTGTGGTGAACCGCTGGGTGGT
TGGTTATACCGGCAAGAGTCCGTGGATAATAACTACCCGGAATGTGACATTTGTGAAGCACTGA

Supplementary Fig. S3. The pairing of the UpRoi1 to the *flgL* (A, B) and *oppA* (C, D) mRNA fragments. (A, C). Binding visualized by the IntaRNA program. Hybridization energy (HE) was calculated by IntaRNA software and equals -23.9 and -18.5 kcal/mol for UpRoi1-*flgL* mRNA and UpRoi1-*oppA* mRNA complexes, respectively, thus indicating high binding ability in both cases. In (C, D), the sequences of *flgL* and *oppA* genes with UpRoi1 binding sites highlighted in blue and Rho binding sites of Rho-dependent terminators T4_Rho and T5_Rho marked in green.



Supplementary Fig. S4. Precursor electronic spectra of UpRoi1 (in black), 5'-UTR *roi* mRNA (in green - panel A), and *flgL* mRNA (in blue – panel B) solutions. Electronic spectra were recorded to establish the predictions for the interaction investigation.



Supplementary Fig. S5. UV-Vis spectra of Tris biological buffer solutions obtained for the (A) UpRoi1- 5'-UTR *roi* mRNA and (B) UpRoi1- *flgL* mRNA pairs (without excluding the dissolution effect) together with parameters for spectroscopic effects observed in the form of intensity changes (hyperchromicity) at 260 nm.

Supplementary Table S2. The list and characteristics of target binding sites identified bioinformatically for UpRoi1 sRNA in the genome of *E. coli* bacteria NC_002695.2

Number	DNA sequence of the binding site	Position in <i>E. coli</i> genome (NC_002695.2)	Gene/locus_tag	Name of the product	Accession number	Hybridization energy [kcal/mol]* calculated by IntaRNA software	Seed position calculated by IntaRNA software**	Interaction of UpRoi1 with the target binding site visualized by IntaRNA software
1	GATGCTTCGGTTCGATGGTGATAGGACAT	1499773-1499802	<i>flgL</i>	flagellar hook-filament junction protein	NP_309488.1	-23.9	2 -- 8	<pre> 3 30 5' -GA UCGCG G U UGCU UUCGAUG UGA AGGACAU ACGA AAGUUAC ACU UCCUGUA 3' -UA CUUAA C C-5' 28 2 </pre>

Supplementary Table S2. The list and characteristics of target binding sites identified bioinformatically for UpRoi1 sRNA in the genome of *E. coli* bacteria NC_002695.2

2	TGGTGTGCGTTTTCACCTGGTGAAAGGCGT	1023517-1023546	<i>ybjO</i>	inner membrane protein	NP_308965.1	-19.7	2 -- 8	<pre> 1 30 5' - G UGCG CUG A AC AC AAAAGU CACU UCCUGUA 3' -UA G UU UA C C-5' 28 2 </pre>
3	GAAAGTTGAATTTTCTATTGAGCAGGGTCA	1230103-1230132	<i>cspG</i>	cold-shock protein	NP_309172.1	-19.4	21 -- 27	<pre> 5 30 5' -GAAA UAU CAG U GUUGAAUUUUC UGAG GG CA CGACUUAAAAG ACUC CC GU 3' -UAA UUAC U U AC-5' 27 3 </pre>

Supplementary Table S2. The list and characteristics of target binding sites identified bioinformatically for UpRoi1 sRNA in the genome of *E. coli* bacteria NC_002695.2

4	GATTGCTGCCTCTTCATTGTGGAAGAAAA	1745365-1745394	<i>oppA</i>	oligopeptide ABC transporter periplasmic binding protein	NP_309770.2	-18.5	24 -- 30	<pre> 2 26 5' -G CCUC U A AAAA-3' AUUGCUG UUCA UGUG GA GA UAACGAC AAGU ACAC CU CU 3' - UUAA U U C GUAC-5' 30 5 </pre>
5	ATCTTGCTGATCTCAATATGAAAGGTATG	3024529-3024558	<i>narP</i>	two-component regulatory system response regulator NarP	NP_311109.1	-14.4	22 -- 28	<pre> 4 25 5' -AUC C UA AA GUAUG-3' UUGCUGGAU UCAA UG AG AACGACUUA AGUU AC UC 3' -U AA AC UC CUGUAC-5' 29 7 </pre>

Supplementary Table S2. The list and characteristics of target binding sites identified bioinformatically for UpRoi1 sRNA in the genome of *E. coli* bacteria NC_002695.2

6	ATTGCTGGCATTCTTACTACTGCGGACA	2654371-2654400	<i>yedA</i>	amino acid exporter for phenylalanine	NP_310724.1	-14.2	24 -- 30	<pre> 1 5' - GCA CUAC CGC A-3' AUUGCUG UUUUUA UG GGAC UAACGAC AAAAGU AC CCUG 3' - UU UAC UCU UAC-5' 30 4 </pre>
7#	CCTGCTGGAATTTTCGGGCTGGAGACAT	1569973-1570002	ECs_1547	phage minor tail protein	NP_309574.1	-24.6	3 -- 9	<pre> 3 5' -CC U GGGGC UGCUUGAU UUUC UG GAGGACAU ACGACUUA AAAG AC CUCCUGUA 3' -UA 28 30 2 </pre>

Supplementary Table S2. The list and characteristics of target binding sites identified bioinformatically for UpRoi1 sRNA in the genome of *E. coli* bacteria NC_002695.2

8#	ATATGCCGGATTTTGAGCGTGAGGATGACT	1962565-1962594	ECs_1986	phage tail assembly protein	NP_310013.1	-14.5	18 -- 24	<pre> 4 28 5' -AUA C GAGC G U CU-3' UGC GGAAAAU GUGA GA GA ACG CUAAAA CACU CU CU 3' -UA A GUUA C GUAC-5' 28 5 </pre>
9#	ATTGCTGAATTTTCAATGTGAGGACATG	1264345-1264374	untranslated region between ECs_1199 and ECs_1200	phage antirepressor Ant phage antirepressor Roi	NP_309226.1 NP_309227.1	-49.3	5 -- 11	<pre> 1 30 5' - AUUGCUGAAUUUCAAUGUGAGAGGACAUG UAACGACUAAAAGUUACACUCUCCUGUAC 3' - 30 1 </pre>

Supplementary Table S2. The list and characteristics of target binding sites identified bioinformatically for UpRoi1 sRNA in the genome of *E. coli* bacteria NC_002695.2

10	GTATTGCTGAAATTTCAACGAGAAATGTT	1427425- 1427454	untranslated region between ECs_1370 and ECs_1372	glucosyl-transferase IS1 family transposase InsB	NP_309397.1 NP_309399.2	-20.4	23 -- 29	
11	TTTGTGAAATTTTATGTTGATTTTACT	5395657- 5395686	untranslated region between <i>nanC</i> and <i>fimB</i>	N-acetylneuraminic acid outer membrane channel protein type 1 fimbriae regulatory protein FimB	NP_313297.2 NP_313298.1	-18.8	23 -- 29	

Supplementary Table S2. The list and characteristics of target binding sites identified bioinformatically for UpRoi1 sRNA in the genome of *E. coli* bacteria NC_002695.2

12	GAAGCGTATTTCTCAGCGGGAGAGACAT	183409-183438	untranslated region between <i>mtn</i> and <i>dgt</i>	5'-methylthioadenosine nucleosidase deoxyguanosine triphosphate triphosphohydrolase	NP_308190.1 NP_308191.1	-17.2	4 -- 10	21 30 5' -GAA . . CGCGG C-5' GAGAGGACAU CUCUCCUGUA 3' -UAA . . UUACA 11 2
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* Hybridization energy of the UpRoi1 molecule and the target RNA fragment

** Identified by the program IntaRNA, predicted location of the "seed" region within the UpRoi1 sequence

UpRoi1 binding sites identified in prophage sequence located in the genome of the *E. coli* NC_002695.2

Supplementary Table S3. List of predicted Rho-dependent terminators for *ant-roi* region. Terminators were predicted by RhoTermPredict software.

PREDICTED RHO-DEPENDENT TERMINATOR T1_Rho	
	Genomic sequence of putative RUT site (Coordinates: 599-677, c/g = 1.105263157894737): CTCAACGGGCTTTCCGCAAAGAAACAGACAGCCTTGTATGGCTGTGGGATTATGCCAACCGCTCACAGGCATTGTTC
	The 150 nt long genomic region immediately downstream is CGTGAGTTGTATCCCGCATTAAACTGATTCAGTCTGGCTATTCCGGCATATGCCACGACTACGGCTATAAGTTCTCGTATATCATCGGGAGGGC GAGGGGCGTTTTAATTAATCACACGCGGGATATAGATATTTATGAGCCTGACGGG
	Palindromic sequences found at coordinates 23-26 e 30-33(Sequence: ACTG) (SCORE: 7) Palindromic sequences found at coordinates 48-51 e 59-62(Sequence: ATAT) (SCORE: 6) PAUSE-CONSENSUS present at the coordinates 91-103*
PREDICTED RHO-DEPENDENT TERMINATOR T2_Rho	
	Genomic sequence of putative RUT site (Coordinates: 1219-1297, c/g = 1.3888888888888888): CCGGAATTCACCGCTCGCCTTGTTGACCGCTGGCGAGAACTCGAAGGGGCAACCGCGAAAATACCACAAACCTTTTCT

The 150 nt long genomic region immediately downstream is GAGGCATTGCGCCTTGCGGCCGACCTTGAAGACCAGAAGGCTGAACTGGAGAAACAGCTTGCTCTCGCAGCACCTAAAGTTGAGTTGCCG ATCGCGTTGGCGAGGCCAGCGGAATTTGATTGGAACTTTGCAAAGGTTGTTGGTATT
Palindromic sequences found at coordinates 1-4 e 10-13(Sequence: AGGC) (SCORE: 10) Palindromic sequences found at coordinates 59-62 e 68-71(Sequence: TGCT) (SCORE: 8) Palindromic sequences found at coordinates 92-95 e 100-103(Sequence: TCGC) (SCORE: 10) Palindromic sequences found at coordinates 98-101 e 105-108(Sequence: TGGC) (SCORE: 10)
PREDICTED RHO-DEPENDENT TERMINATOR T3_Rho
Genomic sequence of putative RUT site (Coordinates: 242-320, c/g = 2.666666666666665): AACCTTTCCCCTGTTACCTATAACCAGATCCCCGTAATTACTACTGAACTGTTGGCTCACCTTTACGGAACAAAAATC
The 150 nt long genomic region immediately downstream is AAAAACATTTCTGATAACTTTCTGAACAACACGACGCGATTTCATGCCCGGAAAGCATTACTTTAAAATTGAAAAAACGAATTACGCGAGTTTAAG AACAGACCCGAAACAATCGGGTTAGTTGGTAAAAATGCCCGCTCCCTCATCCTC
Palindromic sequences found at coordinates 42-45 e 53-56(Sequence: ATGC) (SCORE: 9) Palindromic sequences found at coordinates 50-53 e 59-62(Sequence: AAAG) (SCORE: 10) Palindromic sequences found at coordinates 102-105 e 113-116(Sequence: CCCG) (SCORE: 11)

Palindromic sequences found at coordinates 103-106 e 112-115(Sequence: CCGA) (SCORE: 12)
Palindromic sequences found at coordinates 101-105 e 113-117(Sequence: ACCCG) (SCORE: 12)
Palindromic sequences found at coordinates 102-106 e 112-116(Sequence: CCCGA) (SCORE: 13)
Palindromic sequences found at coordinates 101-106 e 112-117(Sequence: ACCCGA) (SCORE: 12)

Supplementary Table S5. Comparison of changes in levels of selected phage and bacterial transcripts in *E. coli* MC1061 ($\Phi 24_B$) [pBAD24_pN_UpRoi1], assessed by RNA-seq technique and RT-qPCR method, relative to control variant *E. coli* MC1061 ($\Phi 24_B$) [pBAD24] at time 210 min after prophage induction with mitomycin C.

Gene	Log2FoldChange $\pm$ SD	
	RNA-seq	RT-qPCR
<i>cI</i>	no changes	no changes
<i>cro</i>	0.25 ± 0.05	3.6 ± 0.08
<i>ant</i>	0.20 ± 0.02	1.95 ± 0.46
<i>roi</i>	0.28 ± 0.14	1.57 ± 0.14
<i>d_ant</i>	0.15 ± 0.04	0.77 ± 0.12
<i>flgL</i>	0.18 ± 0.04	4.43 ± 0.16

Supplementary Table S6. List of predicted Rho-dependent terminators for *flgL* gene sequence. Terminators were predicted by RhoTermPredict software.

PREDICTED RHO-DEPENDENT TERMINATOR T4_Rho	
	Genomic sequence of putative RUT site (Coordinates: 110-188, c/g = 1.9285714285714286): TAACCCTTCAGACGATCCCATTGCTGCATCACAAGCTGTTGTTCTCTCCCAGGCACAGGCGCAAAACAGCCAGTACAC
	The 150 nt long genomic region immediately downstream is GCTGGCACGTACTTTCGCCACTCAAAAAGTGTCAGTGAAGAGAGTGTAAGTCCAGGTCACCACCGCTATCCAGAATGCTCAGGAAAAAAT TGTCTACGCCAGCAATGGCACCTTGAGTGACAATGACCGGGCCTCGCTGGCTACGGA
	Palindromic sequences found at coordinates 32-35 e 43-46(Sequence: CACT) (SCORE: 8) Palindromic sequences found at coordinates 100-103 e 108-111(Sequence: GCCA) (SCORE: 11)
PREDICTED RHO-DEPENDENT TERMINATOR T5_Rho	
	Genomic sequence of putative RUT site (Coordinates: 569-647, c/g = 1.3157894736842106): ACCAGACGGTAGCGCTTCTGAAACCAATCTTTTTGCCATGCTGGATAGCGCCATCGCAGCCCTGAAAACGCCGGTCGC

The 150 nt long genomic region immediately downstream is GGATAGCGAAGCGGATAAAGAAACCGCCGCTGCGGCGTTAGATAAAACCAACCGCGGACTGAAAACTCGCTGAACAATGTCCTGACCGTT CGCGCGGAATTAGGCACGCAGCTGAACGAACTGGAGTCGCTGGATTCATTAGGTAGCGA
Palindromic sequences found at coordinates 24-27 e 33-36(Sequence: CGCC) (SCORE: 10) Palindromic sequences found at coordinates 25-28 e 32-35(Sequence: GCCG) (SCORE: 10) Palindromic sequences found at coordinates 24-28 e 32-36(Sequence: CGCCG) (SCORE: 11) PAUSE-CONSENSUS present at the coordinates 103-115

Supplementary Table S7. The list of DNA oligonucleotides used in the RT-qPCR method and to prepare templates for reactions of *in vitro* transcription.

Primer name	Sequence (5' to 3')
roi_48_F	TAA TAC GAC TCA CTA TAG CTG AAT TTT CAA TGT GAG AGG ACA TGA CAA TG
roi_48_R	ATT TAT CAG CTC ATT CAT TGT CAT GTC CTC TCA CAT TG
flgL_F	TAA TAC GAC TCA CTA TAG ATG TCG GCG GAA CAG AAA GTA TTA AGC AAC AGG TCG ATG CTT CGC G
flgL_R	CGA AAA TTT TGT CGC CCG TAT GTC CTA TCA CCA TCG AAC GCG AAG CAT CGA CCT GTT GC
oppA_F	TAA TAC GAC TCA CTA TAG ATC TGC ATA AAA AGC TGG CGA TTG CTG CCT CTT C
oppA_R	CGT TTA CAC CAA TGT TTTT CTT CCA CAA TGA AGA GGC AGC AAT CGC CAG C
cl_Φ24B_F	TCA CAC GAA GAC CAA AGG CA
cl_Φ24B_R	CGT GGT TGG TGA GGC AAT TC
cro_Φ24B_F	CGA AGG CTT GTG GAG TTA GC
cro_Φ24B_R	GTC TTA GGG AGG AAG CCG TT
ant_Φ24B_F	CGT ATA TCA TCG GGA GGG CG
ant_Φ24B_R	AAG CCT TTC CCA TGC CAG AA
roi_Φ24B_F	GCC GAC CTT GAA GAC CAG AA
roi_Φ24B_R	CAA AAT TCC GCT GGC CTC G
d_ant_Φ24B_F	TGT TTG CTA CCG GGC TGA AT

d_ant_Φ24B_R	CCC TTT GCC TGT ATC AGC CA
flgL_MC1061_F	GAT AAA GAA ACC GCC GCT GC
flgL_MC1061_R	CGA CTC CAG TTC GTT CAG CT
icdA_MC1061_F	TAT GGG TTG GAC TGA AGC GG
icdA_MC1061_R	GCC TTC CAT CAG ACG TTC GA

Z uwagi na objętość i format danych uniemożliwiający ich czytelny wydruk, pliki Excel zawierające:

- 1. Supplementary Table S1.** The sequence of algorithm activities allowing for the identification of target binding sites for UpRoi1 and the list of the obtained results.
- 2. Supplementary Table S4.** The list of differentially expressed genes in the *E. coli* (Φ24_B) [pBAD24_pN_UpRoi1] vs *E. coli* (Φ24_B) [pBAD24] generated in RNA-Seq analysis.

zostały przekazane do oceny w formie elektronicznej jako załącznik (płyta CD) do niniejszej rozprawy doktorskiej, jak również są one dostępne na stronie czasopisma jako suplement do niniejszej publikacji (DOI: 10.1016/j.bbagrm.2026.195135).

Oświadczenia współautorów

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01.04.2026
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OŚWIADCZENIE

Niniejszym oświadczam, iż mój wkład w publikację:

BLOCH, S., LEWANDOWSKA, N., ZWOLENKIEWICZ-NOWAK, J., MAZUR, M., CHYLEWSKA, A., DĄBROWSKA, A. M., MUSIAŁ, N., CZAPLEWSKA, P., WESOŁOWSKI, W., ŁUKASIAK, A., FERENC, M., PIETRZYCKI, M., WĘGLIŃSKA, E., MACH, P., WĘGRZYN, G., OLEJNICZAK, M., & NEJMAN-FALEŃCZYK, B. (2026). UPROI1 - THE PHAGE-DERIVED MICRORNA-SIZE NON-CODING RNA MOLECULE REGULATING BOTH PHAGE AND ITS BACTERIAL HOST DEVELOPMENT. *BIOCHIMICA ET BIOPHYSICA ACTA. GENE REGULATORY MECHANISMS*, 1869(1), 195135. [HTTPS://DOI.ORG/10.1016/J.BBAGRM.2026.195135](https://doi.org/10.1016/j.bbagr.2026.195135)

polegał na: (i) współudziale w opracowaniu metody izolacji i identyfikacji cząsteczek typu mikroRNA fagowego pochodzenia; (ii) współprzygotowaniu biblioteki bardzo małych RNA do sekwencjonowania nowej generacji (NGS); (iii) identyfikacji i określeniu ilości transkryptu UpRoi z wykorzystaniem techniki RT-qPCR podczas spontanicznej i wymuszonej mitomycyną C indukcji profaga Φ_{24B} w komórkach bakterii *Escherichia coli* niosących plazmid pBAD24, pBAD24_UpRoi1 oraz pBAD24_pN_UpRoi1; (iv) określeniu żywotności bakterii *E. coli* niosących plazmid pBAD24, pBAD24_UpRoi1 oraz pBAD24_pN_UpRoi1 po indukcji profaga Φ_{24B} wymuszonej mitomycyną C z wykorzystaniem techniki cytometrii przepływowej; (v) współudziale w analizie wiązania białka LexA do promotora *pN*; (vi) przygotowaniu prób do analiz transkryptomicznych i proteomicznych; (vii) analizie poziomu ekspresji genów w komórkach *E. coli* niosących plazmid pBAD24 oraz pBAD24_pN_UpRoi1 podczas indukcji profaga Φ_{24B} wymuszonej mitomycyną C z wykorzystaniem techniki RT-qPCR; (viii) współudziale w planowaniu doświadczeń i interpretacji uzyskanych wyników; (ix) przygotowaniu rycin; (x) współudziale w przygotowaniu i redagowaniu manuskryptu; (xi) pomocy w przygotowaniu odpowiedzi na uwagi recenzentów.

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OŚWIADCZENIE

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polegał na: (i) współudziale w przygotowaniu konstruktów do nadekspresji cząsteczki UpRoi1; (ii) zbadaniu wpływu cząsteczki UpRoi1 na zmiany w gęstości hodowli bakteryjnej i liczbie uwalnianych cząstek fagowych podczas spontanicznej i wymuszonej dodaniem mitomycyny C indukcji profaga; (iii) współudziale w analizie wpływu cząsteczki sRNA na wydajność procesu lizogenizacji bakterii po infekcji fagiem; (iv) współudziale w identyfikacji z wykorzystaniem metod bioinformatycznych interakcji pomiędzy sekwencją cząsteczki UpRoi1 a potencjalnymi docelowymi miejscami wiązania w genomie faga i bakterii oraz potwierdzenie wiązania się cząsteczki UpRoi1 z 5'-UTR *roi* mRNA oraz mRNA genu *flgL* z wykorzystaniem analizy wiązania RNA za pomocą metody różnicowej migracji kompleksów w żelu poliakrylamidowym; (v) syntezie *in vitro* i oczyszczeniu cząsteczek RNA oraz mRNA wykorzystanych w eksperymentach pomiaru spektrofotometrycznego UV oraz spektroskopii dichroizmu kołowego, potwierdzających bezpośrednie wiązanie sRNA z mRNA; (vi) potwierdzeniu wiązania się białka ProQ do kompleksu sRNA-mRNA; (vii) współudziale w analizie wiązania się białka LexA do promotora *pN*; (viii) wykonaniu eksperymentu analizującego ruchliwość komórek w warunkach nadekspresji cząsteczki UpRoi1; (ix) współudziale w opracowaniu metod badawczych; (x) współudziale w planowaniu doświadczeń i interpretacji uzyskanych wyników; (xi) napisaniu wstępu i współtworzeniu rozdziału poświęconego opisowi materiałów i metod; (xii) przygotowaniu tabel i rycin z wyników przeprowadzonych doświadczeń; (xiii) współudziale w przygotowaniu i redagowaniu manuskryptu; (xiv) pomocy w przygotowaniu odpowiedzi na uwagi recenzentów.

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OŚWIADCZENIE

Niniejszym oświadczam, iż mój wkład w publikację:

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polegał na: pomocy w przeprowadzaniu eksperymentów, jak i analizie danych w zakresie wiązania RNA za pomocą metody różnicowej migracji kompleksów w żelu poliakrylamidowym.

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OŚWIADCZENIE

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polegał na: pomocy w przeprowadzaniu eksperymentów oraz analizie danych w zakresie wiązania RNA za pomocą metody różnicowej migracji kompleksów w żelu poliakrylamidowym.

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OŚWIADCZENIE

Niniejszym oświadczam, iż mój wkład w publikację:

BLOCH, S., LEWANDOWSKA, N., ZWOLENKIEWICZ-NOWAK, J., MAZUR, M., CHYLEWSKA, A., DĄBROWSKA, A. M., MUSIAŁ, N., CZAPLEWSKA, P., WESOŁOWSKI, W., ŁUKASIAK, A., FERENC, M., PIETRZYCKI, M., WĘGLIŃSKA, E., MACH, P., WĘGRZYN, G., OLEJNICZAK, M., & NEJMAN-FALEŃCZYK, B. (2026). UPR011 - THE PHAGE-DERIVED MICRORNA-SIZE NON-CODING RNA MOLECULE REGULATING BOTH PHAGE AND ITS BACTERIAL HOST DEVELOPMENT. BIOCHIMICA ET BIOPHYSICA ACTA. GENE REGULATORY MECHANISMS, 1869(1), 195135. [HTTPS://DOI.ORG/10.1016/J.BBAGRM.2026.195135](https://doi.org/10.1016/j.bbagr.2026.195135)

obejmował:

optymalizację metodologii pomiarów spektroskopowych, w tym przeprowadzenie badań kinetycznych mających na celu określenie czasu niezbędnego do osiągnięcia stanu równowagi dwóch układów oddziałujących dwóch cząsteczek RNA; wykonanie właściwych oznaczeń wraz z ich powtórzeniami; analizę i opracowanie uzyskanych wyników, w tym wyznaczenie wartości stałych wiązania (K_{bind}); a także przygotowanie części manuskryptu obejmujących opis metodologii, prezentację wyników (w tym wykonanie wykresów i zależności pomiędzy parametrami spektralnymi a stężeniami / długościami fal), szczegółową dyskusję oraz podsumowanie dla paragrafów artykułu bezpośrednio związanych z miareczkowaniem spektrofotometrycznymi w zakresie UV.



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Podpisano przez:

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Date / Data: 2026-
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PODPIS

Raport z weryfikacji podpisu

INFORMACJE O DOKUMENCIE:	
Nazwa pliku	Chylewska A_final paper 2-sig.pdf
Data weryfikacji podpisów	2026-03-23 18:59:44

SZCZEGÓŁY WERYFIKACJI:

Agnieszka Chylewska	23.03.2026
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OŚWIADCZENIE

Niniejszym oświadczam, iż mój wkład w publikację:

BLOCH, S., LEWANDOWSKA, N., ZWOLENIKIEWICZ-NOWAK, J., MAZUR, M., CHYLEWSKA, A., DĄBROWSKA, A. M., MUSIAŁ, N., CZAPLEWSKA, P., WESOŁOWSKI, W., LUKASIAK, A., FERENC, M., PIETRZYCKI, M., WĘGLIŃSKA, E., MACH, P., WĘGRZYN, G., OLEJNICZAK, M., & NEJMAN-FALEŃCZYK, B. (2026). UPR01 - THE PHAGE-DERIVED MICRORNA-SIZE NON-CODING RNA MOLECULE REGULATING BOTH PHAGE AND ITS BACTERIAL HOST DEVELOPMENT. BIOCHIMICA ET BIOPHYSICA ACTA. GENE REGULATORY MECHANISMS, 1869(1), 195135. [HTTPS://DOI.ORG/10.1016/J.BBAGRM.2026.195135](https://doi.org/10.1016/j.bbagr.2026.195135)

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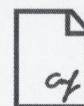


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Podpis: Pozytywny	
Rodzaj certyfikatu	Kwalifikowany
Format podpisu	PAdES-BES
Data złożenia podpisu	2026-03-23 12:18:40
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26.03.2026 16:59:32 GMT+1
Dokument podpisany elektronicznie
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DĄBROWSKA, A. M., MUSIAŁ, N., CZAPLEWSKA, P., WESOŁOWSKI, W., ŁUKASIAK, A., FERENC, M.,
PIETRZYCKI, M., WĘGLIŃSKA, E., MACH, P., WĘGRZYN, G., OLEJNICZAK, M., & NEJMAN-FALEŃCZYK,
B. (2026). UpRo11 - THE PHAGE-DERIVED MICRORNA-SIZE NON-CODING RNA MOLECULE REGULATING
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obejmował:

- optymalizację metodologii pomiarów przeprowadzonych metodą dichroizmu kołowego (CD), w tym analizę wykonanych oznaczeń dwóch układów oddziałujących dwóch cząsteczek RNA w wybranych stechiometriach molowych;
- opracowanie uzyskanych wyników, w tym wyznaczenie wartości stałych wiązania (K_{bind});
- przygotowanie części manuskryptu obejmujących opis metodologii, prezentację wyników (zależności parametrów CD od długości fali w badanym zakresie), szczegółową dyskusję oraz podsumowanie dla paragrafów artykułu bezpośrednio związanych z metodą CD.

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OŚWIADCZENIE

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polegał na: przygotowaniu próbek do analizy metodą spektrometrii mas, rejestracji widm DDA i DIA, przeprowadzeniu analiz bioinformatycznych uzyskanych danych, interpretacji wyników i formułowaniu wniosków końcowych, przygotowaniu rycin oraz pisaniu i redagowaniu manuskryptu.

.....Natalia Musiał.....

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Polegał na: opracowaniu danych ze spektrometrii mas, przygotowaniu materiału do publikacji.

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dr hab. Paulina Czaplewska, prof. UG

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8.04.2026

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Niniejszym oświadczam, iż mój wkład w publikację:

BLOCH, S., LEWANDOWSKA, N., ZWOLENKIEWICZ-NOWAK, J., MAZUR, M., CHYLEWSKA, A., DĄBROWSKA, A. M., MUSIAŁ, N., CZAPLEWSKA, P., WESOŁOWSKI, W., ŁUKASIAK, A., FERENC, M., PIETRZYCKI, M., WĘGLIŃSKA, E., MACH, P., WĘGRZYN, G., OLEJNICZAK, M., & NEJMAN-FALEŃCZYK, B. (2026). UpRoil - THE PHAGE-DERIVED MICRORNA-SIZE NON-CODING RNA MOLECULE REGULATING BOTH PHAGE AND ITS BACTERIAL HOST DEVELOPMENT. BIOCHIMICA ET BIOPHYSICA ACTA. GENE REGULATORY MECHANISMS, 1869(1), 195135. [HTTPS://DOI.ORG/10.1016/J.BBAGRM.2026.195135](https://doi.org/10.1016/j.bbagr.2026.195135)

polegał na: (i) współdziale w przygotowaniu konstruktów do nadekspresji cząsteczki UpRoil; (ii) wykonaniu serii badań wstępnych pozwalających ocenić wpływ cząsteczki UpRoil na rozwój faga i bakterii oraz (iii) współdziale w analizie uzyskanych wyników.

.....
Wesołowski

PODPIS

Aleksandra Lukasiak

.....
IMIĘ I NAZWISKO

08.04.2026

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DATA

Katedra Biologii Molekularnej
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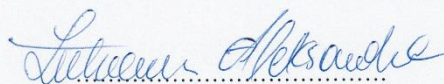
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AFILIACJA

OŚWIADCZENIE

Niniejszym oświadczam, iż mój wkład w publikację:

BLOCH, S., LEWANDOWSKA, N., ZWOLENKIEWICZ-NOWAK, J., MAZUR, M., CHYLEWSKA, A., DĄBROWSKA, A. M., MUSIAŁ, N., CZAPLEWSKA, P., WESOŁOWSKI, W., LUKASIAK, A., FERENC, M., PIETRZYCKI, M., WĘGLIŃSKA, E., MACH, P., WĘGRZYN, G., OLEJNICZAK, M., & NEJMAN-FALEŃCZYK, B. (2026). UpRoi1 - THE PHAGE-DERIVED MICRORNA-SIZE NON-CODING RNA MOLECULE REGULATING BOTH PHAGE AND ITS BACTERIAL HOST DEVELOPMENT. BIOCHIMICA ET BIOPHYSICA ACTA. GENE REGULATORY MECHANISMS, 1869(1), 195135. [HTTPS://DOI.ORG/10.1016/J.BBAGRM.2026.195135](https://doi.org/10.1016/j.bbagra.2026.195135)

polegał na: (i) udziale w przygotowaniu lizatów fagowych do doświadczeń.



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PODPIS



Mariusz Ferenc

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IMIĘ I NAZWISKO

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Instytut Biocybernetyki i Inżynierii Biomedycznej im. Macieja Nałęcz
Camino Science sp. z o.o.

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OŚWIADCZENIE

Niniejszym oświadczam, iż mój wkład w publikację:

BLOCH, S., LEWANDOWSKA, N., ZWOLENKIEWICZ-NOWAK, J., MAZUR, M., CHYLEWSKA, A., DĄBROWSKA, A. M., MUSIAŁ, N., CZAPLEWSKA, P., WESOŁOWSKI, W., ŁUKASIAK, A., FERENC, M., PIETRZYCKI, M., WĘGLIŃSKA, E., MACH, P., WĘGRZYN, G., OLEJNICZAK, M., & NEJMAN-FALEŃCZYK, B. (2026). UpRoi1 - THE PHAGE-DERIVED MICRORNA-SIZE NON-CODING RNA MOLECULE REGULATING BOTH PHAGE AND ITS BACTERIAL HOST DEVELOPMENT. *BIOCHIMICA ET BIOPHYSICA ACTA. GENE REGULATORY MECHANISMS*, 1869(1), 195135. [HTTPS://DOI.ORG/10.1016/J.BBAGRM.2026.195135](https://doi.org/10.1016/j.bbagr.2026.195135)

polegał na współudziale w opracowaniu algorytmu i wyznaczeniu potencjalnych miejsc wiązania dla cząsteczki UpRoi1 z wykorzystaniem narzędzi bioinformatycznych oraz w przygotowaniu ostatecznej wersji manuskryptu.

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PODPIS



Raport z weryfikacji podpisu

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SZCZEGÓŁY WERYFIKACJI:



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MARIUSZ FERENC
03.03.2026 10:59:17 GMT+1
Dokument podpisany elektronicznie
podpisem: Mariusz Ferenc

Mariusz Ferenc

.....

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Polska Akademia Nauk
 Instytut Biocybernetyki i Inżynierii Biomedycznej im. Macieja Nałęcza
 Camino Science sp. z o.o.

.....

AFILIACJA

OŚWIADCZENIE

Niniejszym oświadczam, iż mój wkład w publikację:

BLOCH, S., LEWANDOWSKA, N., ZWOLENIKIEWICZ-NOWAK, J., MAZUR, M., CHYLEWSKA, A., DĄBROWSKA, A. M., MUSIAŁ, N., CZAPLEWSKA, P., WESOŁOWSKI, W., ŁUKASIAK, A., FERENC, M., PIETRZYCKI, M., WĘGLIŃSKA, E., MACH, P., WĘGRZYN, G., OLEJNICZAK, M., & NEJMAN-FALEŃCZYK, B. (2026). UpRoil - THE PHAGE-DERIVED MICRORNA-SIZE NON-CODING RNA MOLECULE REGULATING BOTH PHAGE AND ITS BACTERIAL HOST DEVELOPMENT. *BIOCHIMICA ET BIOPHYSICA ACTA. GENE REGULATORY MECHANISMS*, 1869(1), 195135. [HTTPS://DOI.ORG/10.1016/J.BBAGRM.2026.195135](https://doi.org/10.1016/j.bbagrm.2026.195135)

polegał na współudziale w opracowaniu algorytmu i wyznaczeniu potencjalnych miejsc wiązania dla cząsteczki UpRoil z wykorzystaniem narzędzi bioinformatycznych oraz w przygotowaniu ostatecznej wersji manuskryptu.

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PODPIS

Podpis: Pozytywny	
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Format podpisu	PAdES-BES
Data złożenia podpisu	2026-03-03 06:59:16
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Uwagi	Informacja PAdES: Opatrzono pieczęcią ministra właściwego do spraw informatyzacji w imieniu: : MARIUSZ FERENC, PESEL: 75011012690, PZ ID: Mariusz_Ferenc

Marcin Pietrzycki

.....
IMIĘ I NAZWISKO

23.02.2026

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DATA

Polska Akademia Nauk
Instytut Biocybernetyki i Inżynierii Biomedycznej im. Macieja Nałęcza
Camino Science sp. z o.o.

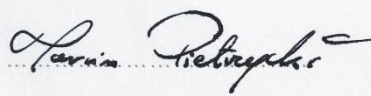
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AFILIACJA

OŚWIADCZENIE

Niniejszym oświadczam, iż mój wkład w publikację:

BLOCH, S., LEWANDOWSKA, N., ZWOLENKIEWICZ-NOWAK, J., MAZUR, M., CHYLEWSKA, A., DĄBROWSKA, A. M., MUSIAŁ, N., CZAPLEWSKA, P., WESOŁOWSKI, W., ŁUKASIAK, A., FERENC, M., PIETRZYCKI, M., WĘGLIŃSKA, E., MACH, P., WĘGRZYN, G., OLEJNICZAK, M., & NEJMAN-FALEŃCZYK, B. (2026). UpRoI1 - THE PHAGE-DERIVED MICRORNA-SIZE NON-CODING RNA MOLECULE REGULATING BOTH PHAGE AND ITS BACTERIAL HOST DEVELOPMENT. *BIOCHIMICA ET BIOPHYSICA ACTA. GENE REGULATORY MECHANISMS*, 1869(1), 195135. [HTTPS://DOI.ORG/10.1016/J.BBAGRM.2026.195135](https://doi.org/10.1016/j.bbagr.2026.195135)

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PODPIS

Emilia Węglińska

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IMIĘ I NAZWISKO

07.04.2026

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DATA

Katedra Biologii Molekularnej
Wydział Biologii
Uniwersytet Gdański

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OŚWIADCZENIE

Niniejszym oświadczam, iż mój wkład w publikację:

BLOCH, S., LEWANDOWSKA, N., ZWOLENKIEWICZ-NOWAK, J., MAZUR, M., CHYLEWSKA, A., DĄBROWSKA, A. M., MUSIAŁ, N., CZAPLEWSKA, P., WESOŁOWSKI, W., ŁUKASIAK, A., FERENC, M., PIETRZYCKI, M., WĘGLIŃSKA, E., MACH, P., WĘGRZYN, G., OLEJNICZAK, M., & NEJMAN-FALEŃCZYK, B. (2026). UpRo11 - THE PHAGE-DERIVED MICRORNA-SIZE NON-CODING RNA MOLECULE REGULATING BOTH PHAGE AND ITS BACTERIAL HOST DEVELOPMENT. BIOCHIMICA ET BIOPHYSICA ACTA. GENE REGULATORY MECHANISMS, 1869(1), 195135. [HTTPS://DOI.ORG/10.1016/J.BBAGRM.2026.195135](https://doi.org/10.1016/j.bbagr.2026.195135)

polegał na: (i) pomocy przy obróbce wyników oraz (ii) edycji spisu literatury ostatecznej wersji manuskryptu.

Emilia Węglińska

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PODPIS

Paulina Mach

.....
IMIĘ I NAZWISKO

27.03.2026

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DATA

Katedra Biologii Molekularnej
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.....
AFILIACJA

OŚWIADCZENIE

Niniejszym oświadczam, iż mój wkład w publikację:

BLOCH, S., LEWANDOWSKA, N., ZWOLENKIEWICZ-NOWAK, J., MAZUR, M., CHYLEWSKA, A., DĄBROWSKA, A. M., MUSIAŁ, N., CZAPLEWSKA, P., WESOŁOWSKI, W., ŁUKASIAK, A., FERENC, M., PIETRZYCKI, M., WĘGLIŃSKA, E., MACH, P., WĘGRZYN, G., OLEJNICZAK, M., & NEJMAN-FALEŃCZYK, B. (2026). UpRoi1 - THE PHAGE-DERIVED MICRORNA-SIZE NON-CODING RNA MOLECULE REGULATING BOTH PHAGE AND ITS BACTERIAL HOST DEVELOPMENT. *BIOCHIMICA ET BIOPHYSICA ACTA. GENE REGULATORY MECHANISMS*, 1869(1), 195135. [HTTPS://DOI.ORG/10.1016/J.BBAGRM.2026.195135](https://doi.org/10.1016/j.bbagrm.2026.195135)

polegał na współudziale w analizie wpływu nadekspresji cząsteczki UpRoi1 na wydajność procesu lizogenizacji bakterii *E. coli* po infekcji fagiem.

Paulina Mach

.....
PODPIS

Grzegorz Węgrzyn

.....
IMIĘ I NAZWISKO

21 marca 2026 r.

.....
DATA

Katedra Biologii Molekularnej
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Uniwersytet Gdański

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AFILIACJA

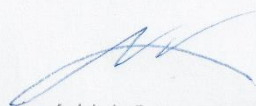
OŚWIADCZENIE

Niniejszym oświadczam, iż mój wkład w publikację:

BLOCH, S., LEWANDOWSKA, N., ZWOLENKIEWICZ-NOWAK, J., MAZUR, M., CHYLEWSKA, A., DĄBROWSKA, A. M., MUSIAŁ, N., CZAPLEWSKA, P., WESOŁOWSKI, W., ŁUKASIAK, A., FERENC, M., PIETRZYCKI, M., WĘGLIŃSKA, E., MACH, P., WĘGRZYN, G., OLEJNICZAK, M., & NEJMAN-FALEŃCZYK, B. (2026). UP011 - THE PHAGE-DERIVED MICRORNA-SIZE NON-CODING RNA MOLECULE REGULATING BOTH PHAGE AND ITS BACTERIAL HOST DEVELOPMENT. *BIOCHIMICA ET BIOPHYSICA ACTA. GENE REGULATORY MECHANISMS*, 1869(1), 195135. [HTTPS://DOI.ORG/10.1016/J.BBAGRM.2026.195135](https://doi.org/10.1016/j.bbagr.2026.195135)

polegał na:

dyskusjach nad koncepcją pracy i interpretacją uzyskanych wyników doświadczalnych oraz wprowadzeniu poprawek do wstępnej wersji manuskryptu.


prof. dr hab. Grzegorz Węgrzyn

.....
PODPIS

Mikołaj Olejniczak

24.03.2026

.....
IMIĘ I NAZWISKO

.....
DATA

Instytut Biologii Molekularnej i Biotechnologii
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Uniwersytet im. Adama Mickiewicza w Poznaniu
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AFILIACJA

OŚWIADCZENIE

Niniejszym oświadczam, iż mój wkład w publikację:

BLOCH, S., LEWANDOWSKA, N., ZWOLENKIEWICZ-NOWAK, J., MAZUR, M., CHYLEWSKA, A., DĄBROWSKA, A. M., MUSIAŁ, N., CZAPLEWSKA, P., WESOŁOWSKI, W., ŁUKASIAK, A., FERENC, M., PIETRZYCKI, M., WĘGLIŃSKA, E., MACH, P., WĘGRZYN, G., OLEJNICZAK, M., & NEJMAN-FALEŃCZYK, B. (2026). UPROI1 - THE PHAGE-DERIVED MICRORNA-SIZE NON-CODING RNA MOLECULE REGULATING BOTH PHAGE AND ITS BACTERIAL HOST DEVELOPMENT. BIOCHIMICA ET BIOPHYSICA ACTA. GENE REGULATORY MECHANISMS, 1869(1), 195135. [HTTPS://DOI.ORG/10.1016/J.BBAGRM.2026.195135](https://doi.org/10.1016/j.bbagr.2026.195135)

polegał na: współudziale w zaplanowaniu eksperymentów oraz analizie danych w zakresie wiązania RNA za pomocą metody różnicowej migracji kompleksów w żelu poliakrylamidowym, jak również w opisanu otrzymanych tą drogą wyników.



Signed by /
Podpisano przez:

Mikołaj Olejniczak

Date / Data:
2026-03-24 12:21

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PODPIS



Raport z weryfikacji podpisu

INFORMACJE O DOKUMENCIE:	
Nazwa pliku	Olejniczak M_2-sig.pdf
Data weryfikacji podpisów	2026-04-09 12:29:48

SZCZEGÓŁY WERYFIKACJI:

Mikołaj Olejniczak	24.03.2026
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IMIĘ I NAZWISKO	DATA
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OŚWIADCZENIE	
Niniejszym oświadczam, iż mój wkład w publikację:	
BIŁOCH, S., LEWANDOWSKA, N., ZWOŁENKIEWICZ-NOWAK, J., MAZUR, M., CHYLEWSKA, A., DĄBROWSKA, A. M., MUSIAŁ, N., CZAPLEWSKA, P., WESOŁOWSKI, W., LUKASIAK, A., FERENC, M., PIETRZYCKI, M., WĘGLIŃSKA, E., MACH, P., WĘGRZYN, G., OLEJNICZAK, M., & NEJMAN-FALEŃCZYK, B. (2026). UPRO1 - THE PHAGE-DERIVED MICRORNA-SIZE NON-CODING RNA MOLECULE REGULATING BOTH PHAGE AND ITS BACTERIAL HOST DEVELOPMENT. BIOCHIMICA ET BIOPHYSICA ACTA. GENE REGULATORY MECHANISMS, 1869(1), 195135. HTTPS://DOI.ORG/10.1016/J.BBAGRM.2026.195135	
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 Signed by / Podpisano przez: Mikołaj Olejniczak Date / Data: 2026-03-24 12:21	
PODPIS	

Podpis: Pozytywny	
Rodzaj certyfikatu	Kwalifikowany
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Numer seryjny certyfikatu	405870549103394775824420180675796712102703250001
Wystawca certyfikatu	Nazwa powszechna: COPE SZAFIR - Kwalifikowany Nazwa organizacji: Krajowa Izba Rozliczeniowa S.A. Kraj: PL Identyfikator organizacji: VATPL-5260300517
Lista CRL wykorzystana do weryfikacji certyfikatu	-
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Uwagi	Weryfikowany podpis został uznany za kwalifikowany. Zgodnie z Art. 25. Punkt 2 rozporządzenia Parlamentu Europejskiego i Rady (UE) nr 910/2014 z 23 lipca 2014 r. kwalifikowany podpis elektroniczny ma skutek prawny równoważny podpisowi własnoręcznemu.

Bożena Nejman-Faleńczyk

09.04.2026 r.

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IMIĘ I NAZWISKO

.....
DATA

Katedra Biologii Molekularnej
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AFILIACJA

OŚWIADCZENIE

Niniejszym oświadczam, iż mój wkład w publikację:

BLOCH, S., LEWANDOWSKA, N., ZWOLENKIEWICZ-NOWAK, J., MAZUR, M., CHYLEWSKA, A., DĄBROWSKA, A. M., MUSIAŁ, N., CZAPLEWSKA, P., WESOŁOWSKI, W., ŁUKASIAK, A., FERENC, M., PIETRZYCKI, M., WĘGLIŃSKA, E., MACH, P., WĘGRZYN, G., OLEJNICZAK, M., & NEJMAN-FALEŃCZYK, B. (2026). UpRoi1 - THE PHAGE-DERIVED MICRORNA-SIZE NON-CODING RNA MOLECULE REGULATING BOTH PHAGE AND ITS BACTERIAL HOST DEVELOPMENT. *BIOCHIMICA ET BIOPHYSICA ACTA. GENE REGULATORY MECHANISMS*, 1869(1), 195135. [HTTPS://DOI.ORG/10.1016/J.BBAGRM.2026.195135](https://doi.org/10.1016/j.bbagra.2026.195135)

polegał na: (i) współudziale w opracowaniu metody izolacji i identyfikacji cząsteczek typu mikroRNA fagowego pochodzenia (ii) zidentyfikowaniu cząsteczki UpRoi1 wśród wyników sekwencjonowania NGS z wykorzystaniem analiz bioinformatycznych, (iii) zainicjowaniu i opracowaniu koncepcji badań nad tą cząsteczką; (iv) określeniu głównych celów badawczych, (v) napisaniu na realizację tych celów projektu i pozyskaniu funduszy z NCN w ramach konkursu Sonata Bis, (vi) kierowaniu projektem i sfinansowaniu przeprowadzonych w ramach projektu badań, których wyniki opublikowano w tej pracy, (vii) nawiązaniu współpracy z naukowcami spoza Katedry i stworzeniu interdyscyplinarnego zespołu (viii) koordynowaniu prac zespołu badawczego oraz nadzorze nad realizacją wszystkich etapów projektu oraz podejmowaniu decyzji o zmianach kierunków badań (ix) współudziale w opracowaniu metod badawczych, (x) opracowaniu głównych założeń manuskryptu i opisanu uzyskanych wyników oraz (xi) poprowadzeniu rozległej dyskusji tej pracy i zaproponowaniu mechanizmu działania cząsteczki UpRoi1.

Jako autor korespondujący odpowiadałam również za kontakt z redakcją czasopisma, proces recenzyjny, wprowadzanie poprawek oraz opracowanie odpowiedzi na uwagi recenzentów. Ponadto czuwałam nad zgodnością pracy z wymogami etycznymi i formalnymi czasopisma oraz nad ostatecznym zatwierdzeniem manuskryptu do publikacji.

B. Nejman-Faleńczyk
.....

PODPIS

Praca nr 4

Application of ultraviolet (UV) and circular dichroism (CD) spectroscopies to study RNA-RNA interactions of regulatory RNAs of bacteriophage origin

Lewandowska, N., Bloch, S., Chylewska, A., Dąbrowska, A. M., Mazur, M., Zwolenkiewicz, J., Wesółowski, W., Łukasiak, A., Węglińska, E., Olejniczak, M., Węgrzyn, G., & Nejman-Faleńczyk, B.

(2026) Methods in Molecular Biology (Clifton, N.J.), 3004, 123–136. (https://doi.org/10.1007/978-1-0716-5084-4_8)

Impact Factor = n/d

Punktacja MNiSW = 70



Chapter 8

Application of Ultraviolet (UV) and Circular Dichroism (CD) Spectroscopies to Study RNA–RNA Interactions of Regulatory RNAs of Bacteriophage Origin

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Abstract

In this chapter, we present the possibility of using ultraviolet (UV) and circular dichroism (CD) spectroscopies to analyze RNA–RNA interactions as an alternative or complementary technique to the classical RNA gel mobility shift assay. We present relevant, detailed protocols and the results obtained for a specific biological model to investigate all three methods. In this model, we studied the binding of small noncoding sRNA molecules of bacteriophage origin to fragments of bacterial mRNAs that were selected based on *in silico* analysis. We conclude that spectroscopic methods can be especially useful in analyzing the interactions of small RNAs with target transcripts and in the experimental validation of their target sites of action.

Key words RNA, RNA interaction, Ultraviolet (UV) spectroscopy, Circular dichroism (CD) spectroscopy, RNA gel mobility shift assay, Gene expression, Regulatory noncoding sRNAs

1 Introduction

RNA–RNA interactions play versatile biological roles, particularly in regulatory processes. Such functional transactions are crucial not only during the translation process [1] but also for regulating gene expression [2]. The latter complex process includes the control of splicing, modulation of mRNA stability, riboswitches, and others. Such RNA-based regulations are crucial for the control of

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biological processes in both prokaryotic and eukaryotic organisms, and they play pivotal roles in human health and disease [3]. Therefore, understanding RNA–RNA interactions is crucial for learning about the various biological processes that nucleic acids regulate. Among these interactions, small and long noncoding RNAs play important roles because of their high abundance and variability in modes of action in the control of cellular processes [4]. Moreover, small RNAs are considered as therapeutic agents, making their importance even more pronounced [5].

The undoubtedly significant role of RNA molecules, mentioned briefly in the preceding paragraph, makes studies on RNA–RNA interactions especially important, as precise biological roles of these nucleic acids cannot be understood without detailed knowledge of the processes in which specific RNA molecules modulate structures and functions of other biologically important molecules. Although the classical methods were based on hybridization of nucleic acids and monitoring the rapidity of their migration of molecules during gel electrophoresis, more advanced techniques are necessary to investigate RNA–RNA interactions in more detail, as summarized recently [6]. Although predictions of such interactions could be performed using bioinformatic tools [7], developing new laboratory techniques is required to verify such theoretical assessments. Methods based on various “omic” analyses provide one possible solution [8], however, still, details of intramolecular transactions need to be determined more directly.

In this study, we investigated spectroscopic methods using ultraviolet light (UV) and circular dichroism (CD) in the analysis of RNA–RNA interactions and compared them with the application of a classic RNA gel mobility shift assay. Small RNA molecules, encoded by bacteriophages (viruses infecting bacterial cells) and playing important roles in the regulation of gene expression, were employed as models (Fig. 1). The protocols of methods are described with special emphasis on the advantages and disadvantages of these methods, and notes are provided indicating details that should be taken into account to avoid technical problems and obtain results that might be ambiguous or difficult to interpret.

2 Materials

Before you start, make sure that you are familiar with the specific requirements related to working with RNA. Always use proper microbiological aseptic techniques. Wear a suitable lab coat and gloves to prevent accidental contamination. After putting on gloves, avoid taking them off and touching contaminated surfaces and equipment. Please remember that RNases can be reintroduced by contact with ungloved hands or unfiltered air. Store all reagents at consistent temperatures according to the manufacturer's

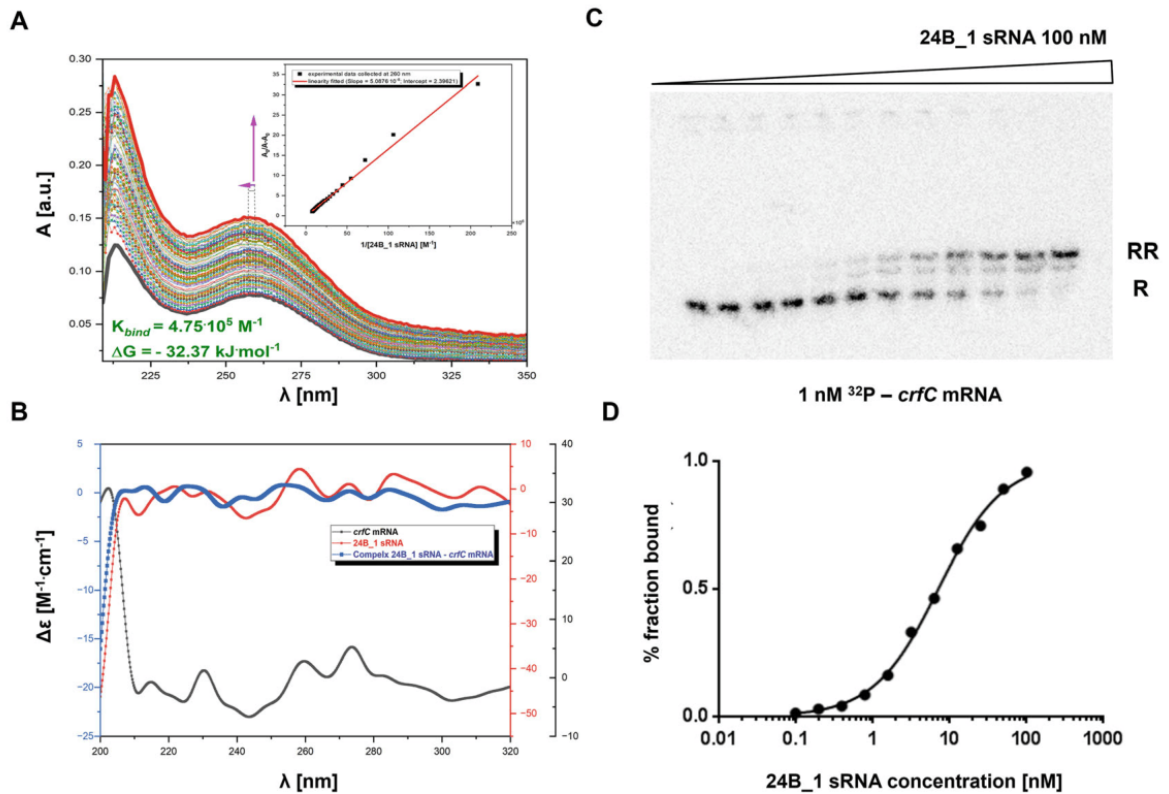


Fig. 1 The example of a direct interaction of phage sRNA with bacterial mRNA studied by UV and CD spectroscopies (**A** and **B**, respectively) and confirmed by RNA gel mobility shift assay (**C**, **D**). Panel **A** shows absorption spectra of *crfC* mRNA obtained in the presence of increasing amounts of 24B_1 sRNA solution with the calculated binding constant K_{bind} of $4.75 \cdot 10^5 M^{-1}$, and the value of Gibbs free energy (ΔG) of -32.37 kJ/mol . The direction of the arrow indicates the hyperchromic effect resulting from intermolecular interactions. Inset shows a linear plot of absorbance against sRNA concentrations. (**B**) CD spectra of the adequate RNA molecules, where 24B_1 is indicated in red, *crfC* mRNA is marked in black, and their complex is presented in blue. (**C**) The binding of the 24B_1 sRNA molecule to the *crfC* mRNA fragment, analyzed by RNA gel mobility shift assay. Unbound ^{32}P -RNA is marked as R, and sRNA-mRNA complex as RR. (**D**) The fitting of the data presented in (**C**) provided as the percentage of fraction bound versus 24B_1 sRNA concentration. Analogous experiments were performed for the second tested pair of UpRoi1 sRNA-*flgL* mRNA

instructions. To avoid RNA degradation, prepare all solutions using ultrapure, diethyl pyrocarbonate (DEPC)-treated water and use RNase decontamination solution.

1. Primers: *crfC*_F, *crfC*_R, *flgL*_F, and *flgL*_R synthesized by Eurofins Scientific based on sequences presented in Table 1 and used at a concentration of $500 \mu\text{M}$ each. Design forward and reverse primers that contain a 20-nt overlapping sequence of a desired DNA fragment (mRNA target molecule). Include in the sequence of forward primer a 17-nt sequence of the T7 RNA polymerase promoter (5' TAATACGACTCACTATA 3'), followed by one or two guanosine/s (see Note 1).

Table 1
DNA sequences of primers

Name	Sequence (5' → 3')
crfC_F	TAATACGACTCACTATAGCTGGAAAGTTATCCCGGTCAATTGACG
crfC_R	CGGGGGTATCCAGTAACGTCAATTGACCGGGATAAC
flgL_F	TAATACGACTCACTATAGATGTCGGCGGAACAGAAAGTATTAAGC AACAGGTCGATGCTTCGCG
flgL_R	CGAAAATTTTGTGCGCCCGTATGTCCTATCACCATCGAACGCGAAG CATCGACCTGTTGC

2. Milli-Q water (RNase-free).
3. 10 × DreamTaq Buffer (Thermo Fisher Scientific).
4. dNTP mix (10 mM each; Thermo Fisher Scientific).
5. DreamTaq DNA Polymerase (Thermo Fisher Scientific).
6. Phenol:chloroform:isoamyl alcohol 25:24:1, pH 8.0: ready-to-use.
7. 3 M sodium acetate (NaOAc): Dissolve 12.3 g NaOAc in Milli-Q water using a magnetic stirrer. Adjust the pH to 5.4 by adding glacial acetic acid. Transfer the solution to a 50 mL measuring cylinder and add Milli-Q water to bring the final volume to 50 mL. Sterilize by filtration using a 0.22-μm pore size PES membrane and then store at room temperature.
8. Ninety-six percent Ethanol: Store at 4 °C.
9. 10 × Transcription buffer, pH 8.0: Dissolve 0.484 g Tris, 0.095 g MgCl₂, 0.029 g NaCl in 4 mL of Milli-Q water. Then, add to the solution 0.1 mL 1 M spermidine. Adjust volume to 5 mL with Milli-Q water. Store this buffer as single-use aliquots at −20 °C.
10. 100 mM Ribo-Nucleotides Set: Separate solutions of ATP, CTP, GTP, and UTP, pH 8.0 (EUR_x).
11. 250 mM DTT: Dissolve 0.386 g DTT in 8 mL of Milli-Q water. Adjust volume to 10 mL of Milli-Q water and then sterilize by filtration using a 0.22-μm pore size PES membrane. Store this solution as single-use aliquots at −20 °C.
12. 1 M magnesium chloride: Dissolve 4.761 g MgCl₂ in 40 mL of Milli-Q water. Adjust volume to 50 mL with Milli-Q water and autoclave for 20 min at 121 °C on liquid cycle.
13. 1 M spermidine: Dissolve 1.45 g spermidine in 10 mL of Milli-Q water and then sterilize by filtration using a 0.22-μm pore size PES membrane. Store this solution as single-use aliquots at −20 °C for no more than 1 month.

14. T7 RNA Polymerase (EUR_X).
15. 0.1 M EDTA, pH 8.0: Dissolve 29.224 g EDTA in Milli-Q water using a magnetic stirrer. Adjust the pH to 8.0 by adding 10 M NaOH. Transfer the solution to a 1000 mL measuring cylinder and add Milli-Q water to bring the final volume to 1000 mL. Autoclave for 20 min at 121 °C on liquid cycle.
16. 8 M urea: Dissolve 48.048 g urea in 70 mL of Milli-Q water using a magnetic stirrer. Transfer the solution to a 100 mL measuring cylinder and add Milli-Q water to bring the final volume to 100 mL. Sterilize by filtration using a 0.22- μ m pore size PES membrane and then store at room temperature.
17. 10% denaturing gel: Mix 14 mL Milli-Q water, 16.8 g urea, 4 mL 10 $\times$ TBE, and 10 mL 40% acrylamide (acrylamide:bis-acrylamide solution 29:1). Heat the mixture to 40 °C and wait until the urea dissolves completely. Then, add 400 μ L 10% APS and 25 μ L TEMED, which catalyze gel polymerization. Mix the solution by gently vortexing and pour the gel into the prepared mold immediately. Allow the gel to polymerize for about 30 min.
18. 10 $\times$ TBE: Dissolve 108 g Tris and 55 g boric acid in 800 mL of Milli-Q water using a magnetic stirrer. Add 40 mL 0.5 M EDTA, pH 8.0. Adjust volume to 1000 mL with Milli-Q water.
19. 0.5 M EDTA, pH 8.0: Dissolve 146.122 g EDTA in Milli-Q water using a magnetic stirrer. Adjust the pH to 8.0 by adding 10 M NaOH. Transfer the solution to a 1000 mL measuring cylinder and add Milli-Q water to bring the final volume to 1000 mL. Autoclave for 20 min at 121 °C on liquid cycle.
20. Forty percent acrylamide:bis-acrylamide solutions 19:1 or 29:1: Ready-to-use.
21. Ten percent APS: Dissolve 0.1 g APS in 1 mL of Milli-Q water. Prepare this mixture fresh each time.
22. *N*, *N*, *N'*, *N'*—Tetramethyl-ethylenediamine (TEMED): Ready-to-use.
23. 1 $\times$ or 0.5 $\times$ TBE buffer: Dilute the 10 $\times$ TBE stock solution by 10 $\times$ or 20 $\times$ in Milli-Q water, respectively.
24. 6 $\times$ DNA Gel Loading Dye (Thermo Fisher Scientific).
25. TLC Silica Gel 60G F₂₅₄ Glass Plate.
26. Elution buffer, pH 8.0: mix 0.09 mL 0.5 M EDTA solution (final concentration of 1 mM) with 4.5 mL 3 M NaOAc (final concentration of 0.3 M) and then adjust the volume to 45 mL with Milli-Q water.
27. Stock solution of 24B_1 sRNA molecule (5'-UAACGUUAA GUUGACUCGGG-3') synthesized by Metabion International AG (at concentration = 282.6 ng/ μ L).

28. Stock solution of *crfC* mRNA (5'-GCUGGAAAGUUAUC CCGGUCAAUUGACGUUACUGGAUACCCCCG-3') obtained by transcription in vitro as described in Subheading 3.1.2 (at concentration = 249.0 ng/ μ L).
29. Stock solution of UpRoi1 sRNA molecule (5'-CAUGUCCUC UCACAU UGAAAAUUCAGCAAU-3') synthesized by Metabion International AG (at concentration = 466.1 ng/ μ L).
30. Stock solution of *flgL* mRNA (5'-GAUGUCGGCGGAAC AGAAAGUAUUAAGCAACAGGUCGAUGCUUCGCGU UCGAUGGUGAUAGGACAUACGGGCGACAAAAUUU UCG-3') obtained by transcription in vitro as described in Subheading 3.1.2 (at concentration = 104 ng/ μ L).
31. RNA Binding buffer: 24 mM Tris-HCl, 150 mM NaCl, 1 mM MgCl₂, 5% glycerol, 100 μ g/mL BSA, pH 7.5.
32. Hamilton's syringe (1.0 mL), volume of the dose: 0.0083 mL.
33. Quartz cuvettes (2 items) with a path length of 1 cm.
34. Quartz cuvettes (2 items) with a path length of 0.1 cm.
35. Six percent non-denaturing gel: Mix 47.4 mL of Milli-Q water, 3 mL 10 $\times$ TBE buffer, and 9 mL 40% acrylamide (acrylamide: bis-acrylamide solution 19:1). Then, add 560 μ L 10% APS and 40 μ L TEMED that catalyze gel polymerization. Mix the mixture by gently vortexing and pour the gel into the prepared mold immediately. Allow the gel to polymerize for about 30 min.

3 Methods

3.1 RNA Molecules Preparation

In this step, Taq polymerase is used to obtain a template for in vitro transcription from overlapping DNA oligonucleotides.

3.1.1 Primer Extension

1. Dissolve the synthesized primers in Milli-Q water at room temperature for approximately 30 min to a final concentration of 500 μ M. Vortex and spin down the tubes briefly in a microcentrifuge.
2. Prepare 200 μ L of the primer extension reaction mixture by adding components to final concentrations of: 1.5 μ M forward primer, 1.5 μ M reverse primer, 1 $\times$ DreamTaq Buffer, 0.2 mM dNTP mix, 0.05 U/ μ L DreamTaq Polymerase. Fill up the sample volume with Milli-Q water to 200 μ L.
3. Mix the contents by slowly inverting the tube. Spin the sample briefly in a microcentrifuge.
4. Perform the primer extension reaction in a thermocycler with the following amplification program: 94 $^{\circ}$ C for 5 min (initial

denaturation stage), followed by 7 cycles of 94 °C for 1 min 15 s (denaturation stage), 55 °C for 1 min 15 s (annealing stage), 72 °C for 2 min (extension stage), and 72 °C for 10 min (final extension stage).

5. Transfer the reaction mixture to a new tube.
6. Extract DNA with 200 µL of cold phenol:chloroform:isoamyl alcohol (25:24:1, pH 8.0) mixture.
7. Vortex the mixture and centrifuge at 7000 RPM for 2 min at 4 °C.
8. Transfer the aqueous phase (~190 µL) to a new tube.
9. Add 19 µL 3 M NaOAc (pH 5.4) and 600 µL of cold 96% ethanol.
10. Incubate the samples for 1 h at –80 °C or overnight at –20 °C.
11. Centrifuge the samples for 40 min at 14,000 RPM at 4 °C. Discard the supernatants and dry the pellets in a vacuum centrifuge for 15–20 min at 30 °C.
12. Resuspend the dried pellets in 50 µL of Milli-Q water.

3.1.2 Transcription In Vitro

In vitro transcription is a technique that allows for the synthesis of RNA molecules from a DNA template using T7 RNA polymerase. As a result of the reaction, we obtain high-purity, defined RNA sequences (mRNA) that can be used, for example, to study RNA-RNA interactions.

1. Dissolve all reagents on ice.
2. Add 10 µL of the DNA template obtained in the primer extension reaction (*see* Subheading 3.1.1) to the PCR tube.
3. Denature the DNA template for 5 min at 90 °C.
4. Refold the DNA template for 10 min at room temperature.
5. Prepare 100 µL of the reaction mixture by adding components to final concentrations of: 1 × Transcription buffer, 2 mM ATP, 2 mM CTP, 2 mM GTP, 2 mM UTP, 25 mM DTT, 18 mM MgCl₂, and 600 U of T7 RNA Polymerase. Fill up the sample volume with Milli-Q water to 100 µL.
6. Incubate the reaction mixture with shaking (300 RPM) for 3 h at 37 °C.
7. Stop the reaction by adding to the sample 33.3 µL 100 mM EDTA (pH 8.0).
8. Add to the sample 13 µL 3 M NaOAc (pH 5.4) and 300 µL 96% ethanol. Precipitate RNA as described in Subheading 3.1.1, **steps** from 3.1.1.10 to 3.1.1.11.
9. Dissolve the pellet in 30 µL 8 M urea at room temperature for 30 min (*see* **Note 2**).

10. Denature RNA sample for 2 min at 90 °C.
11. Prepare a 10% denaturing polyacrylamide gel to purify the RNA sample from the side products of the synthesis by using polyacrylamide gel electrophoresis (PAGE).
12. Pre-run gel without samples for 10 min in 1 x TBE buffer at 10 W.
13. Load samples into separate wells of previously prepared polyacrylamide gel (*see Note 3*).
14. Run electrophoresis for about 50 min at 15 W (*see Note 4*).
15. Stop electrophoresis and wrap the gel in stretch film.
16. Transfer the gel on a silica-coated thin-layer chromatography (TLC) plate and visualize bands of primers via short-wave UV light (260 nm; *see Note 5*).
17. To recover the gel slice that contains RNA, excise bands of appropriate size with scalpels.
18. Place the gel pieces in the separate tubes filled with 500 µL Elution buffer and incubate with shaking (300 RPM) overnight at 4 °C (*see Note 6*).
19. Centrifuge samples and transfer the supernatants into new tubes.
20. Precipitate RNA by adding 3 volumes of cold 96% ethanol.
21. Incubate the samples for 1 h at –80 °C or overnight at –20 °C.
22. Centrifuge the samples for 40 min at 14,000 RPM at 4 °C. Discard the supernatants and dry the pellets in a vacuum centrifuge for 15–20 min at 30 °C.
23. After precipitation, dissolve the RNA pellet in 20–30 µL of Milli-Q water and incubate for 30 min at room temperature.
24. Determine the RNA concentration (ng/µL) at 260 nm on a spectrophotometer. Store the RNA sample at –20 °C for further analysis.
25. Calculate the micromolar concentration of the RNA sample using the formula:

$$C [\mu\text{M}] = (A_{260}/\text{ext.Coeff}) \times 1,000,000$$

where:

- **C [µM]** is the concentration of RNA in µM,
- **A₂₆₀** is the absorbance at 260 nm,
- **ext. Coeff** is the extinction coefficient (also known as the molar absorptivity), a constant specific to the nucleic acid type (this factor may be found using online tools after providing with RNA sequence),
- **1,000,000** is a scaling factor to convert the result from molarity (mol/L) to micromolar (µM), where 1 µM = 1 × 10^{–6} mol/L.

3.2 UV Spectroscopy

The automated UV titration method facilitates quantitative characterization of RNA–RNA interactions by tracking variations in ultraviolet absorbance (at 260 nm) as one RNA molecule is incrementally titrated into a solution containing a complementary RNA particle. The use of an automated titration apparatus ensures precise volumetric delivery and high reproducibility of measurements, enabling accurate determination of the saturation point, the equilibrium binding constant, and key thermodynamic parameters, like free Gibbs energy. This noninvasive technique offers rapid acquisition of binding data, making it particularly well-suited for detailed investigations into RNA structure, interaction kinetics, and molecular recognition events.

This method can be applied to study short RNA oligonucleotides or structured RNAs without introducing fluorescent labels or chemical modifications, preserving the molecules' native structure and dynamics.

1. Calibrate the spectrophotometer (an Evolution 300 spectrophotometer, Thermo Fisher Scientific) using a RNA Binding buffer during the initial stage of the UV measurements to eliminate the signal of its components during the recording of interactions.
2. Verify the initial concentrations of the RNA molecules using spectrophotometric analysis.
3. For **Pair 1**, set [*crfC* mRNA] to 0.12 μM in the cuvette and [24B_1 sRNA] to 0.29 μM in the syringe.
4. For **Pair 2**, set [UpRoi1 sRNA] to 0.33 μM in the cuvette and [*flgL* mRNA] to 0.025 μM in the syringe.
5. Use the computer-controlled automatic microtitrator (e.g., CerkoLab) for titration process.
6. Titrate 0.6 mL of *crfC* mRNA (titrand) with the 24B_1 sRNA solution (titrant).
7. Titrate 0.5 mL of UpRoi1 sRNA (titrand) with the *flgL* mRNA solution (titrant).
8. Maintain UV titration parameters as follows: Allow 120 s between each dose, include a 90-s break, and mix for 30 s at a constant temperature of 298 K.
9. Record the electronic absorption spectra after each titrant addition (*see Note 7*).
10. Examine the stability of the titrand's binding properties toward the titrant. Determine the association/binding constants K_{bind} of the interactions with the titrant molecule based on the variation in absorbance. Apply the Benesi-Hildebrand equation [9] for the calculation:

$$\frac{A_0}{A - A_0} = \frac{\epsilon_G}{\epsilon_{H-G} - \epsilon_G} + \frac{\epsilon_G}{\epsilon_{H-G} - \epsilon_G} \cdot \frac{1}{K_{bind}[TITRANT]}$$

where:

- A_0 and A are the absorbance of the *TITRAND* in the absence and presence of *TITRANT*, respectively;
 - ϵ_G and ϵ_{H-G} are the absorption coefficients of the unbonded *TITRAND* and its adduct with *TITRANT*, respectively.
11. Construct a double reciprocal plot of $A_0/(A - A_0)$ versus $1/[TITRANT]$.
 12. Estimate the binding constant (K_{bind}) based on the linear fit parameters by calculating the ratio of the intercept to the slope [10, 11].
 13. Determine the free Gibbs energy (ΔG) for both systems using the equation below and the known values of the binding constants (K_{bind}).

$$\Delta G = -R \cdot T \cdot \ln(K_{bind})$$

where:

- ΔG —free Gibbs Energy.
 - R —molar gas constant [J/molK].
 - T —temperature [K].
 - K_{bind} —the value of binding constant (formation of **P1** or **P2** adducts).
14. Perform all UV experiments in duplicate, using two independent experimental sets.
 15. Fit all experimental data using analysis software (see **Note 8**).
 16. Use the mean $\pm$ standard deviation (SD) for statistical analysis of the data and for creating graphics (see **Note 8**).

3.3 CD Spectroscopy

Circular dichroism (CD) spectroscopy offers a powerful, non-denaturing, and label-free technique for investigating RNA–RNA interactions in solution. CD is particularly sensitive to changes in the secondary structure of nucleic acids, such as the formation of helices or tertiary motifs, which often accompany RNA complex formation. Upon binding of two RNA molecules, the resulting conformational rearrangements produce characteristic shifts in the CD spectrum, enabling direct detection of interaction events.

Compared to the classical RNA gel mobility shift assay, CD spectroscopy allows real-time monitoring of interactions under near-physiological conditions. It is especially valuable for identifying subtle structural transitions, determining binding stoichiometries, and assessing the thermodynamic stability of RNA complexes.

Furthermore, CD can be employed to validate and complement gel shift assay results, particularly in cases where complexes are unstable, transient, or difficult to resolve by gel-based methods.

By providing both qualitative and quantitative insights into RNA folding and hybridization, CD spectroscopy serves as a robust tool for RNA structural biology and interaction studies and represents a versatile addition to the experimental toolkit for RNA-focused research.

1. Prepare and measure the probes: Prepare solutions of the titrant, titrand, and their adduct. Measure each sample using circular dichroism (CD) spectroscopy. Ensure that the concentrations are optimized for the spectropolarimeter's detection range.
2. Record CD spectra: Acquire CD spectra at binding buffer (see point 2.32) using a spectropolarimeter (Jasco J-815).
 - (a) Set the detection range to 200–320 nm.
 - (b) Use a spectral bandwidth of 0.2 nm.
 - (c) Set the scan speed to 50 nm/min.
 - (d) Maintain constant temperature and ensure cuvettes are clean and free of contaminants.
3. Generate absorbance bands: Using the UV spectra obtained during the final step of spectrophotometric titration, generate absorbance bands for both adducts.
 - (a) Compare with reference spectra.
 - (b) Note spectral shifts indicative of adduct formation.
 - (c) Identify characteristic maxima and minima in absorbance.
4. Correct the ellipticity (θ) values from the CD spectra for the buffer signal and convert the values to $\Delta\epsilon$ in $[\text{mol}\cdot\text{dm}^{-3}\cdot\text{cm}^{-1}]$ using the following relationship:

$$\Delta\epsilon = \frac{\theta}{32,980 \cdot C \cdot L \cdot N}$$

where:

- θ is the measured (raw) CD amplitude in [mdeg]
- C is the sample concentration in $[\text{mol}\cdot\text{dm}^{-3}]$.
- L is the cuvette path length $L = 0.1$ [cm].
- N is the number of nucleotides of the RNA.

3.4 RNA Gel Mobility Shift Assay

When working with radioactive isotopes, always follow institutional safety guidelines and wear appropriate personal protective equipment, including gloves and lab coats. Use designated areas to work and plexiglass protection shields. Monitor contamination regularly

with Geiger counters or wipe tests. Dispose of radioactive waste according to approved procedures. Minimize exposure time to the radioactive samples, maximize distance, and use proper shielding to reduce radiation risk.

1. Prepare ^{32}P -labeled RNA according to an established protocol [12].
2. Store the RNA sample as a 200 nM solution at $-20\text{ }^{\circ}\text{C}$ for further analysis.
3. Allow RNA samples to dissolve on ice.
4. In two separate tubes, mix the ^{32}P -RNA and unlabeled RNA with the $5\times$ RNA Binding buffer and Milli-Q water to a final concentration of 2 nM and 200 nM of RNA, respectively.
5. Denature RNA samples for 2 min at $90\text{ }^{\circ}\text{C}$ and cool down at room temperature.
6. Prepare the desired range of 11 concentrations of unlabeled RNA (from 200 nM to 0.195 nM) by serial dilution in the $1\times$ RNA Binding buffer (ratio 1:1).
7. Mix unlabeled RNA dilutions in a ratio of 1:1 with 2 nM ^{32}P -labeled RNA in the $1\times$ RNA Binding buffer. For the sample without unlabeled RNA mix the $1\times$ RNA Binding buffer with 2 nM ^{32}P -labeled RNA. The final concentrations were 1 nM of ^{32}P -RNA and 100 nM of unlabeled RNA at the maximum concentration used.
8. Incubate the mixtures for 4 h at room temperature.
9. Prepare a native 6% polyacrylamide gel to visualize RNA–RNA interaction by using PAGE.
10. Pre-run gel without the samples for 10 min in $0.5\times$ TBE buffer at 10 W.
11. Load 5 μL of samples from **step 3.4.8** into separate wells of a previously prepared polyacrylamide gel.
12. Run the gel for 1 h at 10 W in a cold room.
13. Place the gel into the vacuum dryer for approximately 50 min at $70\text{ }^{\circ}\text{C}$ (*see Note 9*).
14. Expose the gel to phosphor screens overnight.
15. Quantify the bound RNA using a phosphorimager with ImageQuant and MultiGauge software.
16. Estimate the equilibrium dissociation constant (K_d) values by using the GraphPad Prism software and the quadratic equation as presented earlier [13].

4 Notes

1. If guanosine is absent from the native sequence of the target DNA fragment, it should be incorporated into the forward primer sequence immediately following the fragment of the T7 RNA polymerase promoter.
2. For more efficient dissolution of the RNA pellet, occasionally tap the tube, followed by a brief spin-down.
3. For visual tracking of the progress of electrophoresis, you can load an equal volume of 6 × DNA Gel Loading Dye into a separate well of the prepared polyacrylamide gel.
4. Monitor the electrophoresis and stop it when bromophenol blue migrates out of the gel.
5. In the UV shadowing technique, the nucleic acid absorbs light and casts a shadow against the fluorescent background, allowing the sample to be visible on the gel.
6. During this step, DNA migrates out of the gel into the Elution buffer.
7. Record the UV absorption spectra using a spectrophotometer. Set the detection range to 200–350 nm, using a spectral bandwidth of 2 nm and a scan speed of 240 nm/min.
8. Eliminate dilution effects associated with the measurement procedure at the preliminary stage of result processing for both UV and CD methods (Origin2022b software, OriginLab Corporation).
9. To avoid contamination of the vacuum dryer, cover the gel from one side with a piece of Whatman paper and from the other side with a layer of stretch film.

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Oświadczenia współautorów

Natalia Lewandowska

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9.04.2026

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OŚWIADCZENIE

Niniejszym oświadczam, iż mój wkład w publikację:

LEWANDOWSKA, N., BLOCH, S., CHYLEWSKA, A., DĄBROWSKA, A. M., MAZUR, M., ZWOLENKIEWICZ, J., WESOŁOWSKI, W., ŁUKASIAK, A., WĘGLIŃSKA, E., OLEJNICZAK, M., WĘGRZYN, G., & NEJMAN-FALEŃCZYK, B. (2026). APPLICATION OF ULTRAVIOLET (UV) AND CIRCULAR DICHROISM (CD) SPECTROSCOPIES TO STUDY RNA-RNA INTERACTIONS OF REGULATORY RNAs OF BACTERIOPHAGE ORIGIN. METHODS IN MOLECULAR BIOLOGY (CLIFTON, N.J.), 3004, 123–136. [HTTPS://DOI.ORG/10.1007/978-1-0716-5084-4_8](https://doi.org/10.1007/978-1-0716-5084-4_8)

polegał na: (i) współudziale w opracowaniu procedury analizy wiązania mRNA-sRNA z wykorzystaniem spektroskopii UV i CD; (ii) przygotowaniu cząsteczek RNA z wykorzystaniem technik wydłużenia startera (ang. primer extension) i transkrypcji *in vitro*; (iii) współudziale w przeprowadzeniu analizy wiązania RNA za pomocą metody różnicowej migracji kompleksów w żelu poliakrylamidowym; (iv) analizie i interpretacji wyników; (v) napisaniu wstępu oraz rozdziałów poświęconych opisowi metod i notatek; (vi) współtworzeniu koncepcji manuskryptu; (vii) współudziale w przygotowaniu ryciny nr 1; (viii) przygotowaniu oraz redagowaniu manuskryptu; (ix) przygotowaniu odpowiedzi na uwagi recenzentów.

Natalia Lewandowska

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LEWANDOWSKA, N., BLOCH, S., CHYLEWSKA, A., DĄBROWSKA, A. M., MAZUR, M., ZWOLENKIEWICZ, J., WESOŁOWSKI, W., ŁUKASIAK, A., WĘGLIŃSKA, E., OLEJNICZAK, M., WĘGRZYN, G., & NEJMAN-FALEŃCZYK, B. (2026). APPLICATION OF ULTRAVIOLET (UV) AND CIRCULAR DICHROISM (CD) SPECTROSCOPIES TO STUDY RNA-RNA INTERACTIONS OF REGULATORY RNAs OF BACTERIOPHAGE ORIGIN. METHODS IN MOLECULAR BIOLOGY (CLIFTON, N.J.), 3004, 123–136. [HTTPS://DOI.ORG/10.1007/978-1-0716-5084-4_8](https://doi.org/10.1007/978-1-0716-5084-4_8)

polegał na: (i) współpracowaniu metodyki; (ii) współtworzeniu koncepcji manuskryptu; (iii) współudziale w przygotowaniu i redagowaniu manuskryptu; (iv) pomocy w przygotowaniu odpowiedzi dla recenzentów.

Sylwia Bloch
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PODPIS

Agnieszka Chylewska

23.03.2026

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Katedra Chemii Bionieorganicznej
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Niniejszym oświadczam, iż mój wkład w publikację:

LEWANDOWSKA, N., BLOCH, S., CHYLEWSKA, A., DĄBROWSKA, A. M., MAZUR, M., ZWOLENKIEWICZ, J., WESOŁOWSKI, W., ŁUKASIAK, A., WĘGLIŃSKA, E., OLEJNICZAK, M., WĘGRZYN, G., & NEJMAN-FALEŃCZYK, B. (2026). APPLICATION OF ULTRAVIOLET (UV) AND CIRCULAR DICHROISM (CD) SPECTROSCOPIES TO STUDY RNA-RNA INTERACTIONS OF REGULATORY RNAs OF BACTERIOPHAGE ORIGIN. METHODS IN MOLECULAR BIOLOGY (CLIFTON, N.J.), 3004, 123–136. [HTTPS://DOI.ORG/10.1007/978-1-0716-5084-4_8](https://doi.org/10.1007/978-1-0716-5084-4_8)

obejmował:

optymalizację metodologii pomiarów spektroskopowych, w tym przeprowadzenie badań kinetycznych mających na celu określenie czasu niezbędnego do osiągnięcia stanu równowagi układu oddziałujących dwóch cząsteczek RNA; wykonanie właściwych oznaczeń wraz z ich powtórzeniami; analizę i opracowanie uzyskanych wyników, w tym wyznaczenie wartości stałych wiązania (K_{bind}); a także przygotowanie części manuskryptu obejmujących opis metodologii, prezentację wyników (w tym wykonanie wykresów i zależności pomiędzy parametrami spektralnymi a stężeniami / długościami fal), szczegółową dyskusję oraz podsumowanie dla paragrafów artykułu bezpośrednio związanych z miareczkowaniem spektrofotometrycznymi w zakresie UV.



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Agnieszka Chylewska
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Date / Data: 2026-
03-23 12:17

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PODPIS

Raport z weryfikacji podpisu

INFORMACJE O DOKUMENCIE:	
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Data weryfikacji podpisów	2026-03-23 18:56:15

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Agnieszka Chylewska IMIĘ I NAZWISKO Katedra Chemii Bionieorganicznej Wydział Chemii Uniwersytet Gdański AFILIACJA	23.03.2026 DATA
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Niniejszym oświadczam, iż mój wkład w publikację:

LEWANDOWSKA, N., BLOCH, S., CHYLEWSKA, A., DĄBROWSKA, A. M., MAZUR, M., ZWOLENIEWICZ, J., WESOŁOWSKI, W., LUKASIAK, A., WĘGLIŃSKA, E., OLENICZAK, M., WĘGRZYN, G., & NEJMAN-FALENCZYK, B. (2026). APPLICATION OF ULTRAVIOLET (UV) AND CIRCULAR DICHROISM (CD) SPECTROSCOPES TO STUDY RNA-RNA INTERACTIONS OF REGULATORY RNAs OF BACTERIOPHAGE ORIGIN. METHODS IN MOLECULAR BIOLOGY (CLIFTON, N.J.), 3004, 123–136. [HTTPS://DOI.ORG/10.1007/978-1-0716-5084-4_8](https://doi.org/10.1007/978-1-0716-5084-4_8)

obejmował:

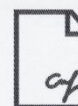
optymalizację metodologii pomiarów spektroskopowych, w tym przeprowadzenie badań kinetycznych mających na celu określenie czasu niezbędnego do osiągnięcia stanu równowagi układu oddziałujących dwóch cząsteczek RNA; wykonanie właściwych oznaczeń wraz z ich powtórzeniami; analizę i opracowanie uzyskanych wyników, w tym wyznaczenie wartości stałych wiązania (K_{bind}); a także przygotowanie części manuskryptu obejmujących opis metodologii, prezentację wyników (w tym wykonanie wykresów i zależności pomiędzy parametrami spektralnymi a stężeniami / długościami fal), szczegółową dyskusję oraz podsumowanie dla paragrafów artykułu bezpośrednio związanych z pomiarami spektrofotometrycznymi w zakresie UV.



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Podpis: Pozytywny	
Rodzaj certyfikatu	Kwalifikowany
Format podpisu	PAdES-BES
Data złożenia podpisu	2026-03-23 12:17:33
Podpis zawiera znacznik czasu	Brak
Certyfikat podpisującego / składającego pieczęć	Nazwa powszechna: Agnieszka Chylewska Nazwa organizacji: Uniwersytet Gdański Kraj: PL
Numer seryjny certyfikatu	718466672265034702462790087905007542203775044271
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Uwagi	Weryfikowany podpis został uznany za kwalifikowany. Zgodnie z Art. 25. Punkt 2 rozporządzenia Parlamentu Europejskiego i Rady (UE) nr 910/2014 z 23 lipca 2014 r. kwalifikowany podpis elektroniczny ma skutek prawny równoważny podpisowi własnoręcznemu.



PODPIS ZAUFANY

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Dokument podpisany elektronicznie
podpisem zaufanym

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23.03.2026

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OŚWIADCZENIE

Niniejszym oświadczam, iż mój wkład w publikację:

LEWANDOWSKA, N., BLOCH, S., CHYLEWSKA, A., **DĄBROWSKA, A. M.**, MAZUR, M., ZWOLENKIEWICZ, J., WESOŁOWSKI, W., ŁUKASIAK, A., WĘGLIŃSKA, E., OLEJNICZAK, M., WĘGRZYN, G., & NEJMAN-FALEŃCZYK, B. (2026). APPLICATION OF ULTRAVIOLET (UV) AND CIRCULAR DICHROISM (CD) SPECTROSCOPIES TO STUDY RNA-RNA INTERACTIONS OF REGULATORY RNAs OF BACTERIOPHAGE ORIGIN. METHODS IN MOLECULAR BIOLOGY (CLIFTON, N.J.), 3004, 123–136. [HTTPS://DOI.ORG/10.1007/978-1-0716-5084-4_8](https://doi.org/10.1007/978-1-0716-5084-4_8)

obejmował:

- optymalizację metodologii pomiarów przeprowadzonych metodą dichroizmu kołowego (CD), w tym analizę wykonanych oznaczeń układu oddziałujących dwóch cząsteczek RNA w wybranych stechiometriach molowych;
- opracowanie uzyskanych wyników, w tym wyznaczenie wartości stałych wiązania (K_{bind});
- przygotowanie części manuskryptu obejmujących opis metodologii, prezentację wyników (zależności parametrów CD od długości fali w badanym zakresie), szczegółową dyskusję oraz podsumowanie dla paragrafów artykułu bezpośrednio związanych z metodą CD.

Aleksandra M. Dąbrowska

Monika Mazur

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31.03.2026r.

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polegał na: pomocy w przeprowadzaniu eksperymentów oraz analizie danych w zakresie wiązania RNA za pomocą metody różnicowej migracji kompleksów w żelu poliakrylamidowym.

Monika Mazur

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Joanna Zwolenkiewicz-Nowak

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21.03.2021
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polegał na: pomocy w przeprowadzaniu eksperymentów w zakresie wiązania RNA za pomocą metody różnicowej migracji kompleksów w żelu poliakrylamidowym.

Joanna Zwolenkiewicz-Nowak
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PODPIS

Wojciech Wesołowski

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8.04.2026

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polegał na współudziale w: (i) analizie wyników i (ii) przygotowaniu rozdziału nr 2.

Wesołowski

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08.04.2026

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polegał na współudziale w: (i) analizie wyników i (ii) przygotowaniu rozdziału nr 2.

Aleksandra Lukasiak

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Emilia Węglińska

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04.04.2026

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polegał na współudziale w: (i) analizie wyników i (ii) przygotowaniu rozdziału nr 2.

Emilia Węglińska

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PODPIS

Mikołaj Olejniczak

24.03.2026

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polegał na: współudziale w opisie metodyki pomiaru wiązania RNA za pomocą monitorowania różnicowej migracji kompleksów w żelu poliakrylamidowym.



Signed by /
Podpisano przez:

Mikołaj Olejniczak

Date / Data:
2026-03-24 12:22

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PODPIS

Raport z weryfikacji podpisu

INFORMACJE O DOKUMENCIE:	
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LEWANDOWSKA, N., BLOCH, S., CHYLEWSKA, A., DĄBROWSKA, A. M., MAZUR, M., ZWOLENKIEWICZ, J., WESOŁOWSKI, W., ŁUKASIAK, A., WĘGLIŃSKA, E., OLEJNICZAK, M., WĘGRZYN, G., & NEJMAN-FALENCZYK, B. (2026). APPLICATION OF ULTRAVIOLET (UV) AND CIRCULAR DICHROISM (CD) SPECTROSCOPIES TO STUDY RNA-RNA INTERACTIONS OF REGULATORY RNAs OF BACTERIOPHAGE ORIGIN. METHODS IN MOLECULAR BIOLOGY (CLIFTON, N.J.), 3004, 123–136. HTTPS://DOI.ORG/10.1007/978-1-0716-5084-4_8	
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PODPIS	

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Certyfikat podpisującego / składającego pieczęć	Nazwa powszechna: Mikołaj Olejniczak Kraj: PL
Numer seryjny certyfikatu	405870549103394775824420180675796712102703250001
Wystawca certyfikatu	Nazwa powszechna: COPE SZAFIR - Kwalifikowany Nazwa organizacji: Krajowa Izba Rozliczeniowa S.A. Kraj: PL Identyfikator organizacji: VATPL-5260300517
Lista CRL wykorzystana do weryfikacji certyfikatu	-
Odpowiedź OCSP wykorzystana do weryfikacji certyfikatu	Status: GOOD ArchiveCutOff: 13 Mar 2017 14:07:47 GMT CN=COPE SZAFIR OCSP Responder OZK62 O=Krajowa Izba Rozliczeniowa S.A. L=Warszawa ST=mazowieckie C=PL - 9 Apr 2026 10:31:42 GMT
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Status weryfikacji	Pozytywny
	Kwalifikowany podpis poprawnie zweryfikowany certyfikatem kwalifikowanym
Uwagi	Weryfikowany podpis został uznany za kwalifikowany. Zgodnie z Art. 25. Punkt 2 rozporządzenia Parlamentu Europejskiego i Rady (UE) nr 910/2014 z 23 lipca 2014 r. kwalifikowany podpis elektroniczny ma skutek prawny równoważny podpisowi własnoręcznemu.

Grzegorz Węgrzyn

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21 marca 2026 r.

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OŚWIADCZENIE

Niniejszym oświadczam, iż mój wkład w publikację:

LEWANDOWSKA, N., BLOCH, S., CHYLEWSKA, A., DĄBROWSKA, A. M., MAZUR, M., ZWOLENKIEWICZ, J., WESOŁOWSKI, W., ŁUKASIAK, A., WĘGLIŃSKA, E., OLEJNICZAK, M., WĘGRZYN, G., & NEJMAN-FALEŃCZYK, B. (2026). APPLICATION OF ULTRAVIOLET (UV) AND CIRCULAR DICHROISM (CD) SPECTROSCOPIES TO STUDY RNA-RNA INTERACTIONS OF REGULATORY RNAs OF BACTERIOPHAGE ORIGIN. METHODS IN MOLECULAR BIOLOGY (CLIFTON, N.J.), 3004, 123–136. [HTTPS://DOI.ORG/10.1007/978-1-0716-5084-4_8](https://doi.org/10.1007/978-1-0716-5084-4_8)

polegał na:

kierowaniu jednym z projektów badawczych, w ramach których powstała ta praca, dyskusjach nad optymalnym przedstawieniem opisanej metody oraz wprowadzeniu poprawek do wstępnej wersji manuskryptu.


prof. dr hab. Grzegorz Węgrzyn

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PODPIS

Bożena Nejman-Faleńczyk

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polegał na: (i) opracowaniu głównych założeń i współudziale w tworzeniu koncepcji manuskryptu oraz zainicjowaniu prac badawczych, (ii) nadzorowaniu całego procesu powstawania i publikacji manuskryptu, (iii) pozyskaniu funduszy na sfinansowanie badań do publikacji i kierowaniu jednym z projektów, w ramach którego powstała ta praca, (iv) nawiązaniu współpracy z naukowcami spoza Katedry i koordynowaniu prac interdyscyplinarnego zespołu badaczy, (v) współudziale w przygotowaniu i redagowaniu manuskryptu oraz (vi) koordynowaniu procesu publikacyjnego jako autor korespondujący.

B. Nejman-Faleńczyk

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PODPIS

Działalność naukowa

Publikacje naukowe:

1. Bloch, S., **Lewandowska, N.**, Zwolenkiewicz-Nowak, J., Mazur, M., Chylewska, A., Dąbrowska, A. M., Musiał, N., Czaplewska, P., Wesółowski, W., Łukasiak, A., Ferenc, M., Pietrzycki, M., Węglińska, E., Mach, P., Węgrzyn, G., Olejniczak, M., & Nejman-Faleńczyk, B. (2026). UpRoi1 - the phage-derived microRNA-size non-coding RNA molecule regulating both phage and its bacterial host development. *Biochimica et biophysica acta. Gene regulatory mechanisms*, 1869(1), 195135. <https://doi.org/10.1016/j.bbagr.2026.195135>.
2. **Lewandowska, N.**, Bloch, S., Chylewska, A., Dąbrowska, A. M., Mazur, M., Zwolenkiewicz, J., Wesółowski, W., Łukasiak, A., Węglińska, E., Olejniczak, M., Węgrzyn, G., & Nejman-Faleńczyk, B. (2026). Application of ultraviolet (UV) and circular dichroism (CD) spectroscopies to study RNA-RNA interactions of regulatory RNAs of bacteriophage origin. *Methods in molecular biology* (Clifton, N.J.), 3004, 123–136. https://doi.org/10.1007/978-1-0716-5084-4_8.
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4. **Lewandowska, N.**, Bloch, S., Łukasiak, A., Wesółowski, W., Węgrzyn, G., & Nejman-Faleńczyk, B. (2025). The role of bacteriophage-derived small RNA molecules in bacterial and phage interactions. *Viruses*, 17(6), 834. <https://doi.org/10.3390/v17060834>.
5. Turbant, F., **Lewandowska, N.**, Bloch, S., Wien, F., Chauvet, H., Węgrzyn, G., & Arluison, V. (2025). Hfq influences ciprofloxacin accumulation in *Escherichia coli* independently of ompC and ompF post-transcriptional regulation. *Journal of applied genetics*, 66(2), 449–457. <https://doi.org/10.1007/s13353-025-00945-9>.
6. Wesółowski, W., Łukasiak, A., Bloch, S., Kuligowska, K., Neumann, J., **Lewandowska, N.**, Węglińska, E., Węgrzyn, G., & Nejman-Faleńczyk, B. (2025). Phage endolysins as promising and effective candidates for use against uropathogenic *Escherichia coli*. *Viruses*, 17(4), 560. <https://doi.org/10.3390/v17040560>.

7. Wons, E., Gucwa, K., **Lewandowska, N.**, Wisniewska, A., Kozłowski, L. P., & Mruk, I. (2025). A transcription factor from the cryptic *Escherichia coli* Rac prophage controls both phage and host operons. *Nucleic acids research*, 53(5), gkaf113. <https://doi.org/10.1093/nar/gkaf113>.

8. Necel, A., Dydecka, A., Topka-Bielecka, G., Wesółowski, W., **Lewandowska, N.**, Bloch, S., & Nejman-Faleńczyk, B. (2025). What, how, and why? - anti-EHEC phages and their application potential in medicine and food industry. *Journal of applied genetics*, 66(1), 219–240. <https://doi.org/10.1007/s13353-024-00918-4>.

9. Bloch, S., **Lewandowska, N.**, Wesółowski, W., Łukasiak, A., Mach, P., Nejman-Faleńczyk, B., & Węgrzyn, G. (2024). Analysis of phage regulatory RNAs: sequencing library construction from the fraction of small prokaryotic RNAs less than 50 nucleotides in length. *Methods in molecular biology* (Clifton, N.J.), 2741, 25–34. https://doi.org/10.1007/978-1-0716-3565-0_3.

10. Bloch, S., **Lewandowska, N.**, Zwolenkiewicz, J., Mach, P., Łukasiak, A., Olejniczak, M., Donaldson, L. W., Węgrzyn, G., & Nejman-Faleńczyk, B. (2023). Bacteriophage-encoded 24B_1 molecule resembles herpesviral microRNAs and plays a crucial role in the development of both the virus and its host. *PloS one*, 18(12), e0296038. <https://doi.org/10.1371/journal.pone.0296038>.

11. Bloch, S., **Lewandowska, N.**, Węgrzyn, G., & Nejman-Faleńczyk, B. (2021). Bacteriophages as sources of small non-coding RNA molecules. *Plasmid*, 113, 102527. <https://doi.org/10.1016/j.plasmid.2020.102527>.

Wystąpienia na konferencjach naukowych:

Wystąpienia ustne

1. *UpRoi1 - mała niekodująca cząsteczka RNA pochodzenia fagowego, pełniąca rolę regulatorową w rozwoju zarówno faga, jak i jego gospodarza.*
III Sympozjum Bakteriofagowe 5-7.09.2024, Gdańsk, Polska

Wystąpienia posterowe

1. *The role of small, non-coding RNA molecules in the regulation of repression-antirepression system of phage $\Phi 24_B$.* International Sopot Youth Conference entitled "Where the World is Heading", 11.06.2021, Sopot, Polska
2. *The role of small RNAs molecules in the regulation of gene expression during the development of $\Phi 24_B$ phage in *E. coli* bacteria.* VI Polski Kongres Genetyki, 27-30.06.2022, Kraków, Polska
3. *Znaczenie małych, niekodujących cząsteczek RNA w przebiegu rozwoju bakteriofaga $\Phi 24_B$ w komórkach *Escherichia coli*.* III Kongres Młodej Nauki 6-8.07.2023, Gdańsk, Polska
4. *Znaczenie małych, niekodujących cząsteczek RNA w przebiegu rozwoju bakteriofaga $\Phi 24_B$ w komórkach *Escherichia coli*.* II Sympozjum Bakteriofagowe 7-9.09.2023, Gdańsk, Polska
5. *UpRoi1: a small non-coding RNA of phage origin with dual regulatory roles in phage and host development,* 2nd Polish RNA Biology Meeting, 24-27.06.2025, Poznań, Polska

6. *Rola małych, niekodujących cząsteczek RNA 24B_1 oraz UpRoi1 w regulacji cyklu życiowego bakteriofagów przenoszących geny toksyn Shiga.* IV Sympozjum Bakteriofagowe 4-6.09.2025, Gdańsk, Polska

Otrzymane nagrody:

1. Nagroda “Srebrnej Ezy” za zajęcie II miejsca za wystąpienie posterowe *The role of small RNAs molecules in the regulation of gene expression during the development of $\Phi 24_B$ phage in E. coli bacteria.* VI Polski Kongres Genetyki, 27-30.06.2022, Kraków, Polska

Staże naukowe:

1. Uniwersytet Adama Mickiewicza, Pracownia Biochemii RNA, Poznań, Polska, 20.02. – 31.03.2023 oraz 23.06 – 5.07.2025, opiekun: prof. Mikołaj Olejniczak
2. Synchrotron SOLEIL, Saint-Aubin, Francja, 18-25.03.2024, opiekun: prof. Véronique Arluison

Udział w projektach naukowych w charakterze wykonawcy:

1. „Rola małych niekodujących RNA w regulacji mechanizmu działania systemu represja-antyrepresja związanego z przełączeniem cyklu życiowego fagów przenoszących geny groźnych dla człowieka toksyn” o numerze UMO-2018/30/E/NZ1/00400, SONATA BIS 8, kierownik projektu: dr hab. Bożena Nejman-Faleńczyk
2. „mikroRNA w regulacji rozwoju bakteriofagów” o numerze UMO-2018/29/B/NZ1/00549, OPUS 15, kierownik projektu: prof. dr hab. Grzegorz Węgrzyn
3. „Regulacja metabolizmu Shiga-toksycznych bakterii *Escherichia coli* przez małe niekodujące cząsteczki RNA, fagowego pochodzenia” o numerze 2023/51/D/NZ2/01338, SONATA 19, kierownik projektu: dr Sylwia Bloch
4. „Molekularne podstawy zakłócania transkrypcji poprzez tzw. cross-talk czynników transkrypcyjnych, i jego wpływ na żywotność bakterii” o numerze 2019/35/B/NZ2/00701, OPUS 18, kierownik projektu: prof. dr hab. Iwona Mruk