Supporting Information

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Materials and Methods

Bacterial Strains and Media. *Pseudomonas aeruginosa* strain PA14 and the isogenic *PA0601* and *nagE* transposon insertion mutants were obtained from the PA14 nonredundant transposon mutant library (1) (http://ausubellab.mgh.harvard.edu/cgi-bin/pa14/home.cgi). Transposon insertions were confirmed by PCR. *P. aeruginosa* was routinely cultured in LB Miller broth/agar or morpholinepropanesulfonic acid (Mops)-buffered defined medium (2) containing 0.1% Socransky vitamin solution (3) and 10 mM succinate, hereby referred to as Mops-V. The ability to catabolize *N*-acetylgalactosamine (GalNAc) was tested by growth on Mops-buffered medium with 20 mM GlcNAc as the sole source of carbon. *Escherichia coli* DH5α or *E. coli* XL1-Blue was cultured on LB Miller broth/agar. *Staphylococcus aureus* strain Mu50 (4) was routinely cultured on BHI broth/agar unless specified otherwise. All cultures were grown at 37 °C, shaking at 250 rpm. Antibiotics were used at the following concentrations: ampicillin, 100 μg/mL; carbenicillin, 300 μg/mL for plasmid selection and 150 μg/mL for plasmid maintenance; gentamicin, 50 μg/mL for *P. aeruginosa* transposon mutants.

Screen for GlcNAc-Unresponsive Mutants. For the screen, mutants arrayed in a 96-well plate format were inoculated into Mops-V supplemented with either 1 mM GlcNAc or 1 mM succinate. Strains were grown with shaking at 37 °C to stationary phase and pyocyanin levels estimated based on the intensity of the blue-green pigment of the culture. Those strains showing no change in the amount of pyocyanin produced in the medium containing GlcNAc compared with the medium lacking GlcNAc were selected. All strains identified in this primary screen were subsequently tested for levels of pyocyanin, lysis of Gram-positive bacteria, and growth with GlcNAc as the primary carbon source.

Pyocyanin Analysis. To quantify pyocyanin, *P. aeruginosa* strains were inoculated into Mops-V supplemented with either 1 mM succinate, 1 mM *N*-acetylmuramic acid, 1 mM GalNAc, 1 mM *N*-acetylneuraminic acid, 1 mM *N*-acetylglucosamine, or 330 μg/mL peptidoglycan. Cells were grown to an OD_{600} of 1.5, and pyocyanin was extracted and quantified from 1-mL cultures as previously described (5).

PQS Extraction and Quantification. *Pseudomonas* quinolone signal (PQS) was extracted from *P. aeruginosa* strains after growth in Mops-V supplemented with 1 mM GlcNAc, 1 mM succinate, or 330 μg/mL peptidoglycan. Cells were grown to an OD_{600} of 1.5 and were extracted three times with an equal volume of acidified ethyl acetate (150 μL acetic acid per liter ethyl acetate; Fisher). Extracts were dried under a constant stream of N_2 gas, resuspended in methanol (Fisher), and analyzed by TLC as described previously (6, 7). PQS was identified by comigration with synthetic PQS standards (Syntech) and quantified by creating a standard curve using 150–500 ng PQS. PQS was visualized by fluorescence after excitation with long-wave UV light using a G:Box gel imager (Syngene).

Quantification of Elastase Activity. *P. aeruginosa* strains were assayed for elastase activity as previously described (8). Briefly, cells were grown in Mops-V supplemented with 1 mM GlcNAc, 1 mM succinate, or 330 μg/mL peptidoglycan to an OD_{600} of 1.5. Cell-free supernatants were collected by filtering whole-cell cultures using a 0.45-μm filter (Corning). Supernatants were added to 1 mL of Elastin Congo Red (ECR; Sigma) substrate and incubated at 37 °C for 18 h with shaking. The ECR substrate is composed of 20 mg of Elastin Congo Red suspended in 1 mL buffer [0.1 M Tris·HCl (pH 7.2) and 1 mM $CaCl_2$]. After 18 h, tubes were placed on ice, and the reaction was stopped by addition of 0.1 mL of 0.12 M EDTA. Insoluble ECR substrate was removed by centrifugation at 16,000 × *g* for 1 min and absorbance measurements taken at 495 nm.

Gram-Positive Lysis Assays. Lysis assays were performed by thoroughly swabbing a Brain Heart Infusion plate with an overnight culture of *S. aureus* Mu50. Plates were air dried, followed by placement of a 6-mm filter disk (Whatman) on the agar. Discs were spotted with 5 μL of an overnight culture of *P. aeruginosa* strains, plates were incubated overnight at 37 °C, and zones of clearing measured.

Complementation of the PA0601 Mutant. The *PA0601* gene was PCR amplified from *P. aeruginosa* chromosomal DNA using the primers listed in Table S2. The forward primer was designed such that the ribosome binding site upstream of *PA0601* would be randomized during PCR. This procedure allows for optimization of expression for complementation and has been used by our group successfully in the past (9). The resulting 633-bp product was digested with SmaI and HindIII and ligated into SmaI/HindIII digested pEX1.8 (10). Five colonies of the resulting pEX1.8 plasmid containing pAK601 were pooled and transformed into the *P. aeruginosa PA0601* mutant by magnesium chloride transformation (2). Transformants were screened for increased pyocyanin production after growth on Mops-V supplemented with either 1 mM GlcNAc or 1 mM succinate. The sequence of PCR-amplified *PA0601* was confirmed by standard automated sequencing. The complementing clone of PA0601 possessed a ribosome binding site of 5′-CTGCT-3′ modified from the WT PA0601 with a ribosome binding site of 5′-GGCAG-3′.

Reverse Transcriptase PCR. Total RNA was extracted from crops excised from flies infected with *P. aeruginosa* WT 48 h after infection using RNABee (Tel-Test) as previously described (11). cDNA synthesis was performed using SuperScript II reverse transcriptase as outlined by the manufacturer (Invitrogen). *P. aeruginosa* RNA (100 ng) served as the template for cDNA synthesis using 250 ng of the random primer $(NS)_5$. Twenty nanograms of the resulting purified cDNA was used as template in the subsequent PCR amplification of *pqsA* and *clpX*. Primers used for PCR are listed in Table S2. For visualization, 5 μL of the resulting PCR was subjected to agarose gel electrophoresis and stained with ethidium bromide.

***P. aeruginosa* Oral Infection of *Drosophila*.** *Drosophila melanogaster* were maintained on standard cornmeal molasses agar [1 L contains 8.2% (wt/vol) cornmeal, 2% (wt/vol) Brewer's yeast, 75 mL molasses, and 1.5% (wt/vol) agar with 0.35% propionic acid, and 0.3% tegosept]. The inoculum for oral infection of *Drosophila* was prepared as follows: *P. aeruginosa* strains grown overnight in LB were adjusted to an OD_{600} of 2.0 in 1.5 mL followed by centrifugation at 5000 × *g* for 15 min to pellet cells. The supernatant was discarded and the pellet resuspended in 100 μL of sterile 5% sucrose. The resuspended cells were spotted onto a sterile 21-mm filter (Whatman) that was placed on the surface of 5 mL of solidified 5% sucrose agar in a plastic vial (FlyBase). In some cases, 330 μg/mL *S. aureus* peptidoglycan (Sigma) was added to the sucrose suspension before spotting onto

sterile filter paper discs. The filters were allowed to dry at room temperature for ~30 min before addition of *Drosophila*. To ensure maximum feeding on the discs containing bacteria, male Canton S flies (1–3 d old) were starved for 3 h before being added to vials (12 flies per vial). Flies were anesthetized by placing them on ice throughout the sorting and transferring process. Infection vials were stored at room temperature in a humidity-controlled environment. The number of live flies to start the experiment was documented, and live flies were counted at 24 h intervals.

1. Liberati NT, et al. (2006) An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. *Proc Natl Acad Sci USA* 103(8): 2833–2838.
2. Ausubel F, et al. (1997) *Short Protocols in Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology* (John Wiley & Sons, New York).
3. Mulcahy H, Sibley CD, Surette MG, Lewenza S (2011) *Drosophila melanogaster* as an animal model for the study of *Pseudomonas aeruginosa* biofilm infections *in vivo*. *PLoS Pathog* 7(10):e1002299.
4. Kuroda M, et al. (2001) Whole genome sequencing of meticillin-resistant *Staphylococcus aureus*. *Lancet* 357(9264):1225–1240.
5. Essar DW, Eberly L, Hadero A, Crawford IP (1990) Identification and characterization of genes for a second anthranilate synthase in *Pseudomonas aeruginosa*: Interchangeability of the two anthranilate synthases and evolutionary implications. *J Bacteriol* 172(2):884–900.
6. Mashburn LM, Whiteley M (2005) Membrane vesicles traffic signals and facilitate group activities in a prokaryote. *Nature* 437(7057):422–425.
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9. Korgaonkar AK, Whiteley M (2011) *Pseudomonas aeruginosa* enhances production of an antimicrobial in response to N-acetylglucosamine and peptidoglycan. *J Bacteriol* 193(4):909–917.
10. Pearson JP, et al. (1994) Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. *Proc Natl Acad Sci USA* 91(1):197–201.
11. Jorth P, Whiteley M (2010) Characterization of a novel riboswitch-regulated lysine transporter in *Aggregatibacter actinomycetemcomitans*. *J Bacteriol* 192:6240–6250.

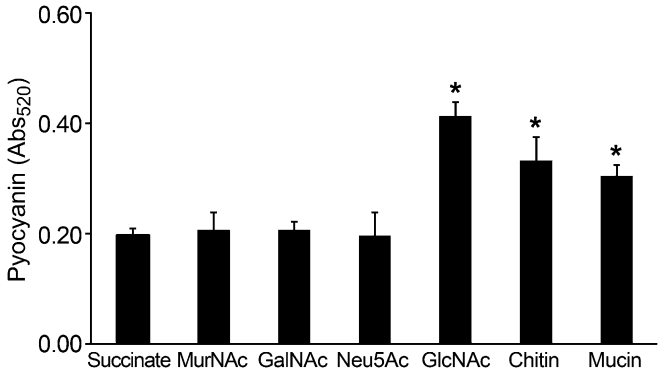

Fig. S1. Induction of pyocyanin is specific to GlcNAc and GlcNAc-containing polymers. Pyocyanin levels produced by WT *P. aeruginosa* grown in Mops-V in the presence of 1 mM succinate, 1 mM *N*-acetylmuramic acid (MurNAc), 1 mM GalNAc, 1 mM *N*-acetylneuraminic acid (Neu5Ac), 1 mM *N*-acetylglucosamine, 1 mg/mL chitin, or 1 mg/mL mucin. *$P < 0.001$ by Student *t* test compared with the no-inducer (succinate) control. Error bars represent SD, $n = 3$.

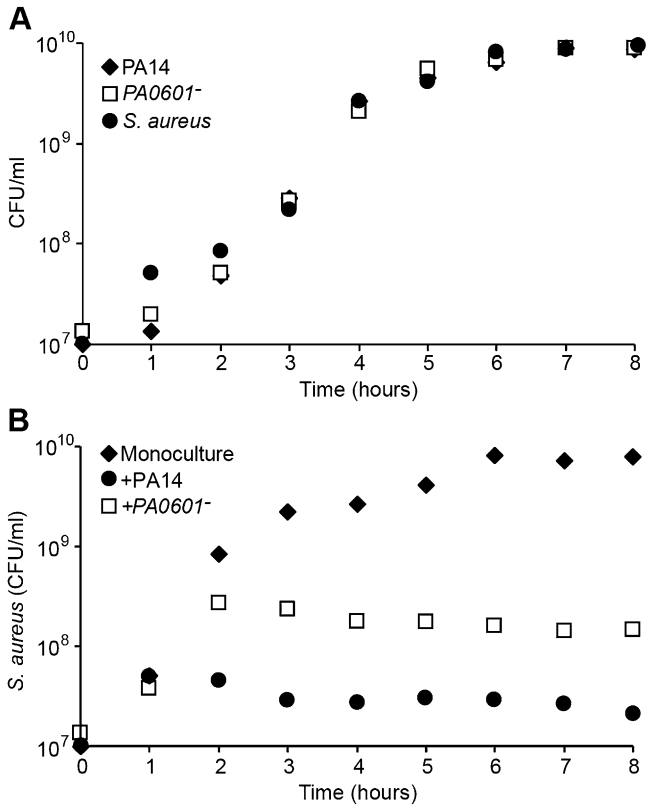

Fig. S2. (*A*) Monoculture growth of WT *P. aeruginosa* (PA14), the *PA0601* mutant (PA0601^{-}), and *S. aureus* in a chemically defined medium (CDM). SD was <10% of the mean for all data points and were not included for the sake of clarity, $n = 3$. (*B*) *P. aeruginosa* PA0601 mutant shows reduced killing of *S. aureus* during in vitro coculture. *S. aureus* was grown in CDM in monoculture and coculture with WT *P. aeruginosa* (PA14) or the *PA0601* mutant (PA0601^{-}) in vitro. Baird-Parker agar was used for enumeration of *S. aureus* cfu/mL. SD was <10% of the mean for all data points and were not included for the sake of clarity, $n = 3$.

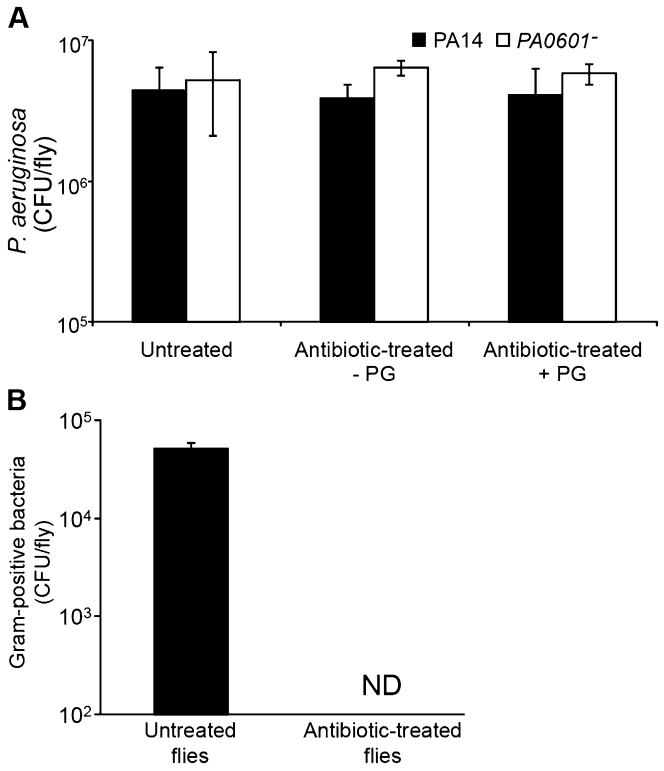

Fig. S3. (*A*) Number of *P. aeruginosa* recovered from *Drosophila* crops is not impacted by inactivation of PA0601, antibiotic treatment of flies, or feeding flies peptidoglycan. *P. aeruginosa* cfu per fly were determined by viable counts on *Pseudomonas* isolation agar. Error bars represent SD, $n = 3$. (*B*) Number of Gram-positive bacteria in the *Drosophila* crop before and after antibiotic treatment. The cfu per fly were enumerated by viable counts on phenylethyl alcohol agar. Error bars represent SD, $n = 3$.

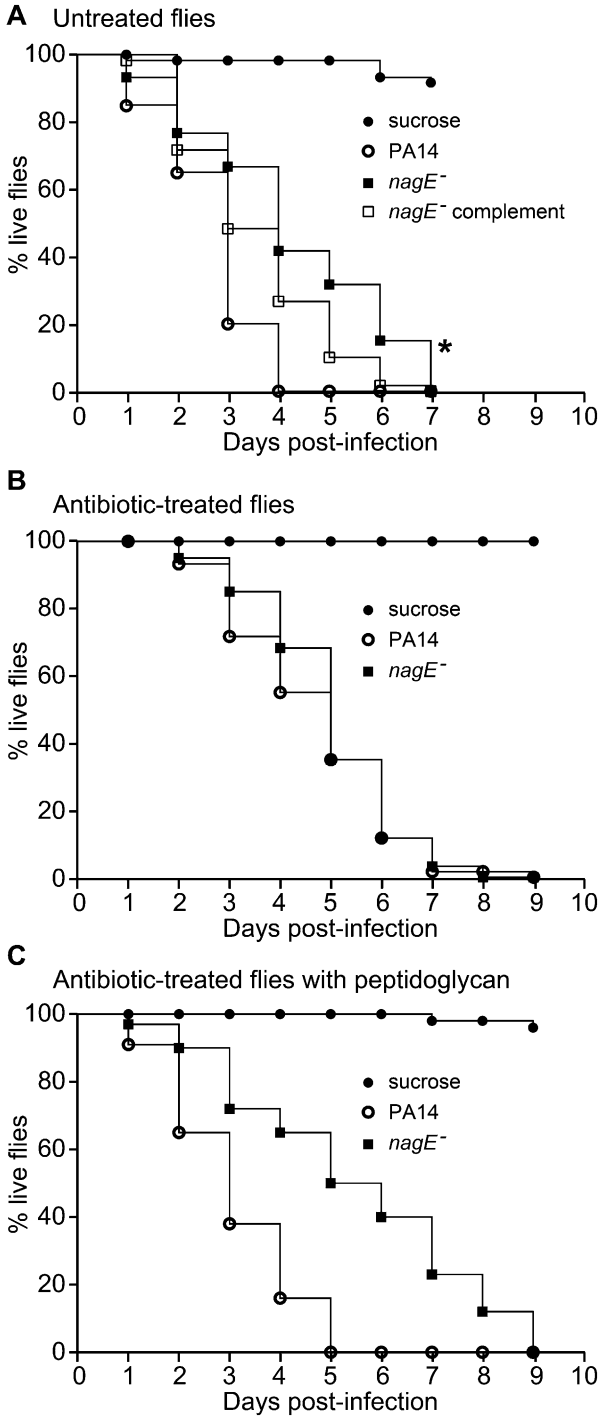

Fig. S4. GlcNAc transport is essential for *P. aeruginosa* virulence in polymicrobial infections. Kaplan-Meier survival curves of (*A*) Antibiotic-untreated *Drosophila* after infection with WT *P. aeruginosa* (PA14), the *nagE* mutant (*nagE*⁻), and the genetically complemented *nagE* mutant (*nagE*⁻ complement). (*B*) Antibiotic-treated flies infected with WT *P. aeruginosa* or the *nagE* mutant. (*C*) Antibiotic-treated flies infected with WT *P. aeruginosa* or the *nagE* mutant in the presence and absence of peptidoglycan. Curves are representative of a minimum of two biological replicates, $n = 60$ for each replicate. $^*P < 0.0001$ by the log–rank test for comparison of percentage survival after infection with WT *P. aeruginosa* compared with infection with *nagE*⁻.

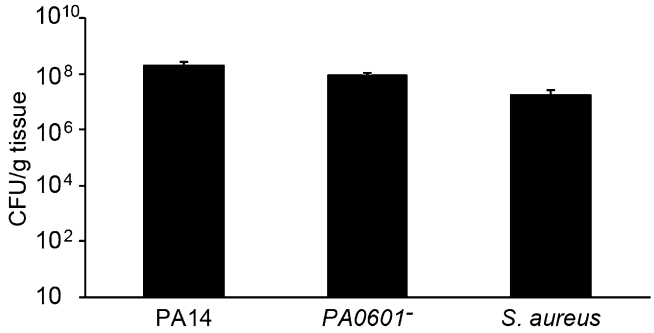

Fig. S5. Monoculture growth yields of WT *P. aeruginosa* (PA14), the *PA0601* mutant (PA0601^{-}), and *S. aureus* after 4 d in the murine wound infection model. Error bars represent SD, $n = 6$. The difference between WT *P. aeruginosa* and PA0601^{-} was not statistically different as determined by Student *t* test, $n = 6$.

Table S1. GlcNAc-unresponsive *P. aeruginosa* mutants

Strain*	Gene*	Description/function*	Growth on GlcNAc[†]	Increased pyocyanin production[‡]	Enhanced *S. aureus* lysis[§]
PA14		WT	+	+	+
PA0266^{-}	*gabT*	4-Aminobutyrate aminotransferase	+	−	+
PA0386^{-}		Putative oxygen-independent coproporhyrinogen III oxidase	+	−	+
PA0601^{-}		Putative two-component response regulator	+	−	−
PA1031^{-}		Hypothetical protein	+	−	+
PA1643^{-}		Putative ATPase	+	−	+
PA1746^{-}		Conserved hypothetical protein	+	−	+
PA1943^{-}		Putative acetyltransferase	+	−	+
PA1945^{-}		Putative sigma-54 dependent regulator	+	−	+
PA2211^{-}		Putative hydrolase	+	−	+
PA2598^{-}		Putative flavin-dependent oxidoreductase	+	−	+
PA3761^{-}	*nagE*	Phosphotransferase system, GlcNAc-specific enzyme IIABC	−	−	−
PA4231^{-}	*pchA*	Salicylate biosynthesis isochromate synthase	+	−	+
PA4319^{-}		Putative membrane protein	+	−	+
PA5138^{-}		Putative ABC-type amino acid transporter, periplasmic component	+	−	+

*From www.pseudomonas.com.

[†]Strains were screened for their ability to catabolize GlcNAc, indicated by + for growth and − for no growth with GlcNAc as the sole carbon source; $n = 3$.

[‡]Increased pyocyanin levels in the presence of GlcNAc or peptidoglycan. +, Increase in pyocyanin production in the presence of GlcNAc or peptidoglycan; −, no change in pyocyanin levels in the presence of GlcNAc or peptidoglycan; $n = 3$.

[§]Lysis of *S. aureus* by *P. aeruginosa* determined using zone of clearing assay [1]. +, A zone of clearing similar to WT *P. aeruginosa*; −, a decreased zone of clearing; $n = 3$.

1. Korgaonkar AK, Whiteley M (2011) *Pseudomonas aeruginosa* enhances production of an antimicrobial in response to N-acetylglucosamine and peptidoglycan. *J Bacteriol* 193(4): 909–917.

Table S2. Strains and plasmids used in this study

Strain or plasmid	Genotype	Reference
Plasmids		
pEX1.8	Broad host range expression vector, Ap^r	1
pAK601	pEX1.8 carrying PA0601	This study
pAKnagE	pEX1.8 carrying *nagE*	This study
E. coli		
DH5α	*endA1 hsdR17 supE44 thi-1 recA1 Δ(lacZYA-argF) U169, deoR* [Φ80d*lac* Δ*(lacZ)M15*]	2
XL1Blue	*endA1 gyrA96(*nal^R*) thi-1 recA1 relA1 lac glnV44 F′[::Tn10 proAB*$^+$ *lacI*^{q} *Δ(lacZ)M15] hsdR17(*r_K^- m_K^+*)*	Stratagene
P. aeruginosa		
PA14	WT	3
PA0601$^-$	PA14 PA0601::Mar2xT7, Gm^r	3
nagE$^-$	PA14 nagE::Mar2xT7, Gm^r	3
S. aureus Mu50	Methicillin-resistant *S. aureus*	4

1. Pearson JP, et al. (1994) Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. *Proc Natl Acad Sci USA* 91(1):197–201.
2. Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY).
3. Liberati NT, et al. (2006) An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. *Proc Natl Acad Sci USA* 103(8):2833–2838.
4. Kuroda M, et al. (2001) Whole genome sequencing of meticillin-resistant *Staphylococcus aureus*. *Lancet* 357(9264):1225–1240.

Table S3. Primers used in this study

Use/primer	Sequence
Complementation of *PA0601*$^-$	
601-F	TCCCCCGGGGAACAGCGTGNNNNNGCAAAG
601-R	CCCAAGCTTACCTGGCGCACAAGCCGCGAG
Complementation of *nagE*$^-$	
nagE-F	TCCCCCGGGGAACAATCGNNNNNNACCGCC
nagE-R	CCCAAGCTTTTGGCCGGGGTCCAGCCCTCC
RT-PCR	
pqsA-F	CAGCCTGGTGGTACGTGAAG
pqsA-R	GCCAGGCGCACGCTGCTCAAC
clpx-F	CCGCGAGGAGGTGCAGGAAGC
clpX-R	GAGGCAACGGTGCCCTCGATG

Restriction sites are underlined.