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The neuroendocrine hormone norepinephrine increases *Pseudomonas aeruginosa* PA14 virulence through the *las* quorum-sensing pathway

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Abstract It has been proposed that the gastrointestinal tract environment containing high levels of neuroendocrine hormones is important for gut-derived *Pseudomonas aeruginosa* infections. In this study, we report that the hormone norepinephrine increases *P. aeruginosa* PA14 growth, virulence factor production, invasion of HCT-8 epithelial cells, and swimming motility in a concentration-dependent manner. Transcriptome analysis of *P. aeruginosa* exposed to 500 μM, but not 50 μM, norepinephrine for 7 h showed that genes involved in the regulation of the virulence determinants pyocyanin, elastase, and the *Pseudomonas* quinolone signal (PQS, 2-heptyl-3-hydroxy-4-quinolone) were upregulated. The production of rhamnolipids, which are also important in *P. aeruginosa* infections, was not significantly altered in suspension cultures upon exposure to 500 μM norepinephrine but decreased on semisolid surfaces. Swarming motility, a phenotype that is directly influenced by rhamnolipids, was also decreased upon 500 μM norepinephrine exposure. The increase in the transcriptional activation of *lasR* but not that of *rhlR* and the increase in the levels of PQS suggest that the effects of norepinephrine are mediated primarily through the *las* quorum-sensing pathway. Together, our data strongly suggest that norepinephrine can play an important role in gut-derived infections by increasing the pathogenicity of *P. aeruginosa* PA14.

Keywords Gut-derived sepsis · Interkingdom signaling

Introduction

Pseudomonas aeruginosa is one of the most common opportunistic pathogens that are present at clinically undetectable levels in the normal gastrointestinal (GI) tract (Alverdy et al. 2000a). However, in critically ill and immunocompromised patients, *Pseudomonas* sp. levels have been shown to increase by as much as 100-fold (Shimizu et al. 2006) leading to the expression of virulence determinants (e.g., PA-I lectin/adhesin; Alverdy et al. 2000a). *P. aeruginosa* has been shown to translocate from the GI tract into the systemic circulation following shock or injury (Alverdy and Chang 2008), and the resultant sepsis rapidly leads to mortality (Schook et al. 1976). In fact, the mere presence of *P. aeruginosa* in the GI tract of critically ill surgical patients has been associated with nearly 70% mortality (Alverdy et al. 2000a). Although the exact mechanisms underlying the increase in *P. aeruginosa* levels and their translocation during stress are largely unknown, it is becoming evident that the interaction of neuroendocrine hormones with bacteria, termed "microbial endocrinology"

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(Lyte 2004), is important in the expression of virulence determinants during infection (Alverdy et al. 2000a).

Norepinephrine (NE), a catecholamine neurotransmitter (stress hormone) that is normally produced in the GI tract through the enteric nervous system (Aneman et al. 1996; Lyte 2004), is one such molecule that is important in GI tract infections. The concentration of NE increases during early sepsis (Hahn et al. 1995), and NE has been shown to stimulate the growth of several Gram-negative and Gram-positive bacteria that are present in the intestinal lumen (Freestone et al. 2008), including *P. aeruginosa* (Freestone et al. 1999). Alverdy et al. (2000a) have correlated the increased NE in the luminal contents of mice after a 30% hepatectomy to increased expression of the *P. aeruginosa* virulence determinant PA-I lectin and gut-derived sepsis. The NE released in the GI tract during stress has also been reported to influence the virulence and infection of other GI tract pathogens. We (Bansal et al. 2007) and others (Chen et al. 2003; Green et al. 2004) have shown that NE increases *Escherichia coli* O157:H7 attachment and colonization to epithelial cells and colonic mucosa, respectively. NE has also been demonstrated to enhance growth, motility, and invasiveness of *Campylobacter jejuni* (Cogan et al. 2007), the expression of the K99 pilus adhesin virulence-related factor in enterotoxigenic *E. coli* (Lyte et al. 1997), *E. coli* O157:H7 virulence gene expression (Bansal et al. 2007; Dowd 2007), and *E. coli* O157:H7 chemotaxis, motility, and biofilm formation (Bansal et al. 2007). Together, these studies indicate that NE plays an important role in the pathogenicity of different bacteria. Although these studies have shown that NE increases virulence and pathogenicity, the mechanisms through which NE impacts virulence of different bacteria are not fully understood.

P. aeruginosa controls the production of many secreted virulence determinants, including elastase, rhamnolipid, pyocyanin, exotoxin A, and catalase (Smith and Iglewski 2003), through the interrelated *las* and *rhl* quorum-sensing (QS) systems (Smith and Iglewski 2003). In addition, the secreted *Pseudomonas* quinolone signal (PQS, 2-heptyl-3-hydroxy-4-quinolone) is also involved in the expression of the virulence determinants rhamnolipids, pyocyanin, and elastase (Diggle et al. 2007). The regulation of PQS is not independent but integrated with the *las* and *rhl* QS (Wade et al. 2005), as the regulator for PQS production (*pqsR*, also known as *mvfR*) is positively regulated by *lasR* and negatively regulated by *rhlR* (Wade et al. 2005).

The goal of this work was to investigate the effect of 50 and 500 μM NE on *P. aeruginosa* virulence and gene expression. The lower concentration (50 μM) was used as representative of NE levels present in the GI tract during homeostasis and has been used in recent studies (Rasko et al. 2008) to investigate the effect of hormones on GI tract pathogens. The higher concentration (500 μM) was used as representative of supraphysiological levels of NE likely to be encountered in the GI tract during catabolic stress (Aneman et al. 1995, 1996). We investigated the effect of NE on the *P. aeruginosa* transcriptome, as well as on the production of different virulence factors, motility, epithelial cell attachment, and invasiveness, barley seed infection, and activation of different QS systems. To our knowledge, this is the first report investigating the molecular basis of alterations in *P. aeruginosa* physiology by NE.

Materials and methods

Bacterial strains, mammalian cell line, and materials

P. aeruginosa PA14 wild type (Liberati et al. 2006) was used for all the experiments. *E. coli* DH5α transformed with plasmids pLP170, pPCS1001, and pPCS1002 containing the *lacZ*, *plasR*::*lacZ*, and *prhlR*::*lacZ* transcriptional fusions, respectively, was kindly provided by Dr. Barbara Iglewski (Pesci et al. 1997). PA14 was grown at 37°C in Roswell Memorial Park Institute (RPMI) 1640 medium (MP Biomedicals, Solon, OH, USA) supplemented with 10% heat-inactivated horse serum (HS; Hyclone, Logan, UT, USA) in all experiments unless indicated otherwise. *L*-(−)-Norepinephrine-(+)-bitartrate (NE) was obtained from EMD Biosciences, La Jolla, CA, USA. The human ileocecal colorectal adenocarcinoma line, HCT-8 (ATCC, Manassas, VA, USA), derived from enterocytes at the junction of the large and small bowel, was grown at 37°C in a 5% CO_2-humidified environment using RPMI 1640 medium supplemented with 10% HS as the growth medium. Elastin-Congo Red obtained from MP Biomedicals was used for the elastase assay. Carbenicillin and gentamicin were obtained from Fisher Scientific (Fair Lawn, NJ, USA) and MP Biomedicals, respectively.

Growth rate measurement

For growth rate measurements, a single colony of PA14 was grown overnight in Luria–Bertani (LB) broth and reinoculated in serum RPMI medium in the presence of 50 and 500 μM NE to an initial turbidity of ~0.05 at 600 nm. The turbidity of the cultures at 600 nm was monitored every hour and the growth rate of the exponentially growing cultures was calculated. Growth curves were obtained in triplicate using three independent cultures, and the statistical significance of specific growth rate was determined using the unpaired Student's *t* test.

Total RNA isolation and microarray analysis

PA14 was grown overnight in LB to a turbidity at 600 nm of ~5.0 and diluted in 100 mL of serum RPMI medium to

an initial turbidity at 600 nm of 0.05. Different concentrations of NE (50 or 500 μM) were added and the cultures were grown for 7 h until late-exponential phase (turbidity at 600 nm of ~0.5, 1.1, and 1.5 with 0, 50, and 500 μM NE, respectively). Cell pellets were prepared and RNA was isolated as described previously (Ren et al. 2004a).

The *P. aeruginosa* Genome Array (Affymetrix, P/N 510596) containing 5,500 of the 5,570 open reading frames of *P. aeruginosa* PA01, was used to analyze changes in the PA14 transcriptome. Complementary DNA synthesis, fragmentation, and hybridizations were as described previously (Gonzalez Barrios et al. 2006). Hybridization was performed for 16 h at 50°C, and the total cell intensity was scaled to an average value of 500. The probe array images were inspected for any image artifact. Background values, noise values, and scaling factors of all the arrays were examined and were comparable. The intensities of polyadenosine RNA control were used to monitor the labeling process. For each binary microarray comparison of differential gene expression, if the gene with the larger transcription rate did not have a consistent transcription rate based on the 13 probe pairs (p value less than 0.05), these genes were discarded. A gene was considered differentially expressed when the p value for comparing two chips was lower than 0.05 (to assure that the change in gene expression was statistically significant and that false positives arise less than 5%), and the expression ratio (between cells treated with NE and control) was greater than 4.0 for 50-μM NE array and 2.0 for 500-μM NE array (based on the standard deviation of fold-change values; Ren et al. 2004b). Gene functions were obtained from http://www.pseudomonas.com/download.jsp. The expression data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/; Edgar et al. 2002) and are accessible through accession no. GSE# 13326.

Quantitative reverse-transcription polymerase chain reaction

Quantitative reverse-transcription polymerase chain reaction (RT-PCR) was performed using a Bio-Rad iCycler (Bio-Rad, CA, USA) real-time PCR unit. Approximately 20 ng of total RNA from the control and NE-treated PA14 was used for each reverse-transcription reaction. Gene sequences were downloaded from http://www.pseudomonas.com/search.jsp and gene-specific primers were used for *plcB*, *lasR*, *lasA*, *pvdS*, *rhlR*, *pilC*, and *proC* (housekeeping control; Table 1). Threshold cycle numbers were calculated using the MyiQ software (Bio-Rad), and PCR products were verified using agarose electrophoresis. RT-PCR experiments were performed thrice (i.e., three experimental replicates) using the same RNA sample used for the microarray analysis.

Table 1 Gene name and its corresponding primer sequence used for qRT-PCR; relative change in expression of the genes determined by microarray (50 μM NE and 500 μM NE) and qRT-PCR at 7 h

PA#	Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Fold change (50 μM NE vs. no NE) 7h		Fold change (500 μM NE vs. no NE) 7h	
				Microarray	qRT-PCR	Microarray	qRT-PCR
PA0026	*plcB*	ACTACACCTCGTACTGGCACTT	TTCAGCTCGCGGTTGTAGAGAT	−2.3	−3.4	4.0[a]	7.1[a]
PA1430	*lasR*	TAAGGACAGCCAGGACTACGAGAA	TGGTAGATGGACGGTTCCCAGAAA	1.2	1.3	1.0	1.5
PA1871	*lasA*	ACCCGAAAGTGTTGTTGACCCT	TTCCTCGAAACCGTAGTAGCGT	1.2	−1.4	6.5[a]	4.7[a]
PA2426	*pvdS*	ATGTGGTCCAGGATGCGTTCTT	TATTTCTGTTCGAGCGCCTGCT	−7.5[a]	−9.8[a]	−2.1[a]	−3.4[a]
PA3477	*rhlR*	AATTTGCTCAGCGTGCTTTCCGTG	TGGGTCAGCAACTCGATCATGCAA	−1.1	−1.5	1.7	1.3
PA4527	*pilC*	TTTCCATGCGCACCACCAATGT	ATCGGCTCCATCAACGTTGTCA	−2.3	−2.8	2.8[a]	1.7
PA0393	*proC*	CAGGCCGGGCAGTTGCTGTC	GGTCAGGCGCGAGGCTGTCT	–	–	–	–

[a] Significant changes in gene expression (greater than fourfold for 50-μM NE array and twofold for 500-μM NE array)

Virulence factor assays

PA14 cells were grown in serum RPMI with 50 or 500 μM NE for 8, 12, 16, and 24 h. At each time point, pyocyanin was extracted from the cell-free culture supernatant into the aqueous phase as described previously (Ueda and Wood 2008). The pyocyanin concentration was normalized to the cell density (turbidity at 600 nm). The PA14 *phzM* mutant was used as the negative control.

Elastase activity in PA14 cultures exposed to 50 or 500 μM NE was determined as described previously (Ohman et al. 1980). The elastase activity was normalized with the cell density (turbidity at 600 nm) to determine elastase activity per cell. The PA14 *lasI* mutant was used as the negative control for elastase production.

PQS was extracted from control and NE-treated PA14 cultures as described previously (Attila et al. 2008b) and measured using a thin-layer chromatography (TLC) assay (Gallagher et al. 2002). Synthetic PQS (Syntech Solution, San Diego, CA, USA) was used as a standard, and the PA14 *pqsA* mutant was used as the negative control. PQS levels were determined by imaging the TLC plate using a VersaDoc 3000 imaging system (Bio-Rad, Hercules, CA, USA).

The pyoverdine concentration was determined by measuring the absorbance of the culture supernatant at 405 nm and normalizing with the cell density (turbidity at 600 nm) as described previously (Stintzi et al. 1998). The PA14 *pvdF* mutant was used as the negative control for pyoverdine production.

The rhamnolipid assay was adapted from Ohman et al. (1980). PA14 cells were grown in serum RPMI with and without 50 and 500 μM NE. After 8, 12, 14, and 24 h, 1 mL of the cell suspension was centrifuged at 10,000×g for 2 min, and rhamnolipids were extracted into the aqueous phase as described previously (Attila et al. 2008b). The absorbance of the aqueous layer was recorded using the orcinol colorimetric assay at 495 nm and normalized by the cell density (turbidity at 600 nm). The PA14 *rhlR* mutant was used as the negative control for rhamnolipid production. All virulence factor assays were performed in triplicates using three independent cultures.

Swimming and swarming motility

The swimming motility assay was adapted from Bearson and Bearson (2008). Briefly, a single colony of PA14 was grown overnight in LB and subcultured at 1:100 in LB medium and grown to a turbidity of ~1.0 at 37°C. NE (50 and 500 μM) was added to 0.3% agar (Difco Laboratories, Detroit, MI, USA) in serum-free RPMI medium. Serum was not included in the plates because of difficulties in preparing serum RPMI agar plates. Instead, 10 μM $FeCl_3$ (Acros Organics, NJ, USA) was added to the medium as Bearson and Bearson (2008) have shown that iron is required for observing NE-mediated changes in phenotype (e.g., swimming motility) in the absence of serum-derived iron. The size of the motility halos was measured after 24 h. Five motility plates were used for each concentration of NE, and the experiment was repeated with three independent cultures (total of 15 plates per NE concentration). The diameter of the transparent zone surrounding the motility halo in PA14 control plates was determined by adding 50 μL of methylene blue (Fisher Scientific, Fair Lawn, NJ, USA) to the edge of the clear zone and by tracing the dye until it covered the border of the entire zone (Caiazza et al. 2005).

Swarming motility was performed as described previously (Overhage et al. 2008). Briefly, fresh BM2 swarm agar plates containing 10 μM $FeSO_4{\cdot}7H_2O$ and supplemented with 0.1% casamino acids (Difco Laboratories, Detroit, MI, USA) and 0.5% bacto agar (Difco Laboratories, Detroit, MI, USA) were used. PA14 were grown overnight in LB, reinoculated at 1:100 in LB medium, and grown to a turbidity of ~1.0 at 37°C. NE (50 and 500 μM) was added to the BM2 swarm agar plates, and the swarming motility pattern was observed after 24 h. The concentration of iron source in BM2 agar was increased to 100 μM and 1 mM $FeSO_4{\cdot}7H_2O$, and the swarming motility pattern of PA14 was observed after 24 h. Motility agar plates were freshly prepared and dried for 3 h prior to the experiment. Five motility plates per NE concentration were used, and the experiment was repeated with three independent cultures (total of 15 plates per NE concentration).

Epithelial cell attachment and invasion assay

Adhesion of PA14 on epithelial cells and its invasion into epithelial cells was determined as described by Bansal et al. (2007) and Fleiszig et al. (1997), respectively. Low-passage HCT-8 cells were seeded into standard 24-well tissue culture plates (Corning Inc., Corning, NY, USA) and grown to ~80% confluency. HCT-8 cell monolayers were washed twice with sterile phosphate-buffered saline (PBS) to remove unattached cells, and the growth medium was replaced with antibiotic-free medium containing 10% heat-inactivated HS. PA14 (turbidity at 600 nm of ~0.8) and NE (50 or 500 μM) were added to wells and incubated for 3 h. Loosely attached PA14 were removed by washing the wells thrice with sterile PBS.

For the adhesion assay, HCT-8 cells were lysed in the wells with 0.1% Triton X-100 and the cell suspension vigorously mixed prior to enumeration of bacteria. For the invasion assay, fresh RPMI medium with 200 μg/mL gentamicin (a concentration which completely killed PA14 suspension cells) was added to each well after removal of

loosely attached PA14 cells. After a 2-h incubation to kill attached (extracellular), *P. aeruginosa*, HCT-8 cells were washed once with PBS and lysed with 0.1% Triton X-100. Bacterial counts for both the adhesion and invasion assays were enumerated by plating the appropriate dilutions of the lysate on LB agar plates. Colonies were counted after a 24-h incubation at 37°C. The experiment was repeated thrice with independent HCT-8 cultures.

Barley seed pathogenicity

The barley seed pathogenicity assay (Attila et al. 2008a) was used to assess *P. aeruginosa* virulence. Briefly, an overnight culture of *P. aeruginosa* PA14 was reinoculated into LB medium at 37°C and grown to a turbidity at 600 nm of ~1. The cells were washed once with sterilized distilled water and twice with sterile Hoagland solution (Shim et al. 2000) and then resuspended to a turbidity at 600 nm of 1.00 ±0.03. Fifteen sterilized barley seeds were placed in beakers containing 10 mL of Hoagland solution with the appropriate concentration of NE and/or PA14 at 25°C with gentle shaking (200 rpm). After 3 days, the number of germinated seeds was counted in each beaker. The experiment was performed with two independent cultures in triplicate (total of 90 seeds per condition).

β-Galactosidase reporter assay

PA14 with the reporter plasmids pLP170 (control with promoterless *lacZ*), pPCS1001 (*plasR*::*lacZ*), and pPCS1002 (*prhlR*::*lacZ*; Pesci et al. 1997) were introduced into PA14 by electroporation. PA14 reporter strains were grown overnight in LB supplemented with 100 μg/mL carbenicillin and reinoculated in two flasks containing 25 mL of serum RPMI medium at 1:100 dilution. NE (50 and 500 μM) was added into the flasks, and the culture was assayed for β-galactosidase activity at 25°C for 4, 5.5, 6.5, and 7.5 h as previously described (Miller 1972). The PA14 cells resuspended in ice-cold TEP buffer (50 mM Tris–HCl [pH 7.4], 5 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonyl fluoride) were sonicated for 30 s twice with an interval of 15 s to lyse the cells. Significant differences between experimental groups were determined using a Student's *t* test at a level of significance of $p<0.05$ or lower.

Results

Effect of NE on *P. aeruginosa* PA14 growth

The effect of NE on PA14 growth was initially determined in order to design subsequent experiments for investigating the effect of NE on PA14 virulence. Cultures of PA14 were treated with 50 and 500 μM NE in serum RPMI medium, and the turbidity at 600 nm was monitored. The addition of 50 and 500 μM NE increased the specific growth rate of PA14 by ~9% and 50%, respectively (0.35±0.04 and 0.49± 0.03 h^{-1} with 50 and 500 μM NE, respectively, compared to 0.32±0.06 h^{-1} for the untreated control). The maximum turbidity at 600 nm reached in the presence of 50 and 500 μM NE was 1.75±0.02-fold and 2.43±0.07-fold greater than untreated controls (Fig. 1).

Differential gene expression in planktonic cells upon exposure to NE

Whole-transcriptome analysis was used to investigate the molecular basis underlying the effect of NE on PA14 gene expression, specifically its effect on the expression of genes involved in the production and regulation of virulence factors. Exposure to 50 μM NE for 7 h (corresponding to late-exponential phase of growth in Fig. 1) significantly altered the expression of 184 genes as compared to the untreated control. Of these, 128 genes were induced fourfold to 209-fold, while 56 genes were repressed fourfold to 42-fold (Supplemental Table I). The genes that were increased in expression upon exposure to 50 μM NE included those of nitrate metabolism (*narGHIJK1K2*; induced 13- to 32.0-fold) that are involved in nitrate assimilation and respiration and genes related to molybdenum cofactor synthesis (*moeA1B1CDE*; induced fivefold to 209-fold) that are involved in nitrogen metabolism (Garzon

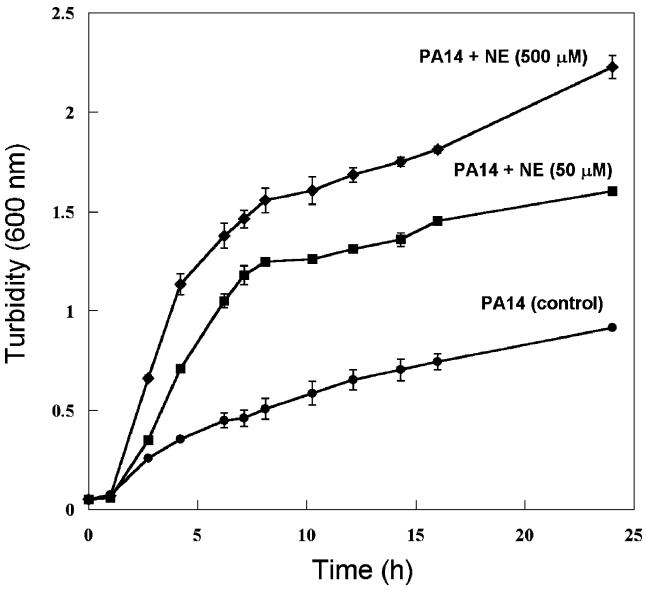

Fig. 1 Effect of NE on growth. *P. aeruginosa* PA14 was grown in RPMI medium at 37°C for 24 h in the presence of 50 and 500 μM NE. The turbidity at 600 nm was measured at different time points. Data shown are mean turbidity ± one standard deviation and are from three independent cultures

Table 2 Partial list of differentially expressed genes in suspension cells of PA14 grown in serum RPMI medium at 37°C for 7 h with 50 and 500 μM of NE

		Fold change[a]		
Locus tag	Gene name	NE vs. control (50 μM)	NE vs. control (500 μM)	Description
Quorum-sensing controlled genes				
PA0026	*plcB*	−2.3	*4.0*	Phospholipase C
PA0044	*exoT*	−1.4	*2.1*	Exoenzyme T
PA0996	*pqsA*	1.0	1.9	Probable coenzyme A ligase
PA0997	*pqsB*	1.1	*2.6*	Beta-keto-acyl carrier protein synthase
PA0998	*pqsC*	1.1	1.1	Beta-keto-acyl carrier protein synthase
PA0999	*pqsD*	1.2	1.7	3-Oxoacyl-[acyl-carrier-protein] synthase III
PA1000	*pqsE*	1.1	*4.3*	Quinolone signal response protein
PA1003	*mvfR*	−1.4	*3*	Transcriptional regulator MvfR
PA1130	*rhlC*	−1.3	*2.8*	Rhamnosyltransferase 2
PA1148	*toxA*	*−8.0*	1.2	Exotoxin A precursor
PA1246	*aprD*	*−4.0*	1.2	Alkaline protease secretion protein AprD
PA1247	*aprE*	*−2.1*	*−2.6*	Alkaline protease secretion protein AprE
PA1249	*aprA*	−1.4	*−2.1*	Alkaline metalloproteinase precursor
PA1710	*exsC*	−1.3	*2.1*	Exoenzyme S synthesis protein C precursor
PA1712	*exsB*	−1.1	*3.7*	Exoenzyme S synthesis protein B
PA1712	*exsA*	−1.2	*2.1*	Transcriptional regulator ExsA
PA1871	*lasA*	1.2	*6.5*	LasA protease precursor
PA1898	*qscR*	2.0	*2.5*	Quorum-sensing control repressor
PA1901	*phzC2*	1.1	*2.5*	Phenazine biosynthesis protein PhzC
PA1902	*phzD2*	1.1	*2.0*	Phenazine biosynthesis protein PhzD
PA1903	*phzE2*	1.0	*2.3*	Phenazine biosynthesis protein PhzE
PA1904	*phzF2*	−1.1	*3.3*	Probable phenazine biosynthesis protein
PA3095	*xcpZ*	1.0	*3.7*	General secretion pathway protein M
PA3097	*xcpX*	−1.1	*2.6*	General secretion pathway protein K
PA3099	*xcpV*	1.0	*2.8*	General secretion pathway protein I
PA3100	*xcpU*	−1.1	*2.8*	General secretion pathway outer membrane protein H precursor
PA3101	*xcpT*	−1.1	*2.5*	General secretion pathway protein G
PA3102	*xcpS*	1.1	*2.8*	General secretion pathway protein F
PA3103	*xcpR*	1.1	*2.0*	General secretion pathway protein E
PA3477	*rhlR*	−1.1	1.7	Transcriptional regulator RhlR
PA3479	*rhlA*	−1.2	1.7	Rhamnosyltransferase chain A
PA3724	*lasB*	1.2	*2.0*	Elastase LasB
PA4209	*phzM*	−1.9	1.3	Probable phenazine-specific methyltransferase
PA4210	*phzA1*	−1.5	−1.2	Probable phenazine biosynthesis protein
PA4211	*phzB1*	1.3	1.3	Probable phenazine biosynthesis protein
PA4217	*phzS*	−2.5	1.1	Flavin-containing monooxygenase
Pyoverdine				
PA2254	*pvcA*	1.0	1.2	Pyoverdine biosynthesis protein PvcA
PA2255	*pvcB*	−1.2	1.4	Pyoverdine biosynthesis protein PvcB
PA2256	*pvcC*	1.3	1.6	Pyoverdine biosynthesis protein PvcC
PA2396	*pvdF*	*−8.6*	−1.2	Pyoverdine synthetase F
PA2397	*pvdE*	*−12.1*	−1.2	Pyoverdine biosynthesis protein pvdE
PA2398	*fpvA*	*−9.2*	1.5	Ferripyoverdine receptor
PA2399	*pvdD*	*−8.6*	*2.3*	Pyoverdine synthetase D
PA2400	*pvdJ*	*−7.5*	*1.3*	PvdJ

Table 2 (continued)

Locus tag	Gene name	Fold change[a] NE vs. control (50 μM)	NE vs. control (500 μM)	Description
PA2426	*pvdS*	*−7.5*	*−2.1*	Sigma factor PvdS
PA4168	*fpvB*	*−12.1*	*2*	Second ferric pyoverdine receptor FpvB
Pyochelin				
PA4221	*fptA*	−1.9	*2.5*	Fe(III)-pyochelin outer membrane receptor precursor
PA4224	*pchG*	−1.2	−1.7	Pyochelin biosynthetic protein PchG
PA4225	*pchF*	−1.3	−1.6	Pyochelin synthetase
PA4226	*pchE*	−1.3	−1.5	Dihydroaeruginoic acid synthetase
PA4227	*pchR*	−2.5	−1.6	Transcriptional regulator PchR
PA4228	*pchD*	−1.1	−1.9	Pyochelin biosynthesis protein PchD
PA4229	*pchC*	−1.2	−1.6	Pyochelin biosynthetic protein PchC
PA4230	*pchB*	1.1	−1.5	Salicylate biosynthesis protein PchB
PA4231	*pchA*	1.0	−1.4	Salicylate biosynthesis isochorismate synthase
Motility				
PA3115	*fimV*	1.1	*2.1*	Motility protein FimV
PA4526	*pilB*	−1.3	1.7	Type 4 fimbrial biogenesis protein PilB
PA4527	*pilC*	−2.3	*2.8*	Still frameshift fimbrial biogenesis protein PilC
PA4528	*pilD*	−1.1	*3*	Type 4 prepilin peptidase PilD
PA1079	*flgD*	−1.1	*2.3*	Flagellar basal-body rod protein FlgD
PA1080	*flgE*	−1.1	1.4	Flagellar hook protein FlgE
PA1087	*flgL*	−1.1	*2.1*	Flagellar hook-associated protein type 3 FlgL
PA1099	*fleR*	−1.1	*4.3*	Two-component response regulator

Complete data for the 50- and 500-μM DNA microarrays are available using GEO series accession number GSE 13326

[a] Most significant changes (greater than fourfold for 50-μM NE array and twofold for 500-μM NE array) are shown in italics

et al. 1992). Expression of the heme acquisition protein *HasAp* decreased by 42-fold, while genes involved in pyoverdine siderophore biosynthesis and metabolism (*pvdADEFGJLNO*) were repressed fivefold to 15-fold (Table 2), respectively, in NE-exposed cultures. In addition, a few genes involved in virulence such as *toxA* (exotoxin A), *aprD* (alkaline protease), and *sodM* (superoxide dismutase) were also repressed by eightfold, fourfold, and 12-fold, respectively. However, genes involved in the production of other virulence determinants (e.g., elastase, alkaline protease, PQS, and rhamnolipids) were not significantly altered upon exposure to 50 μM NE.

Exposure of PA14 to a higher concentration of NE for 7 h significantly induced the expression of 287 genes and repressed the expression of 50 genes (Supplemental Table II). The data showed that 34 genes were altered in expression in both PA14 exposed to 50 or 500 μM NE (Supplemental Table III). The commonly regulated genes primarily included those related to nitrogen metabolism and respiration. In addition, the expression of several QS-controlled genes associated with virulence was significantly altered in PA14 exposed to 500 μM NE (Table 2). These included the elastase synthesis genes *lasA* and *lasB*, which were induced 6.5-fold and twofold, respectively, phospholipase gene *plcB* which was induced fourfold, rhamnolipid synthesis-related gene (*rhlG*) which was repressed 6.5-fold in the NE-treated cells, and phenazine biosynthesis (pyocyanin) genes (*phzCDEF*) which were induced twofold to 3.3-fold. Other significantly induced virulence factor production and infection genes included those involved in flagellar synthesis (*flgD* and *flgL*; 2.3-fold and 2.1-fold), type IV fimbriae (*pilC* and *pilD*; 2.8-fold and threefold), PQS synthesis (*pqsABE* and *mvfR*; induced 1.9-fold to fourfold), exoenzyme S regulation and production (*exsABC*; twofold to fourfold), and exoenzyme T (*exoT*, twofold). Also included are genes involved in alkaline protease secretion (*aprA* and *aprE*, 2.1-fold and 2.6-fold) and the Fe(III)-pyochelin outer membrane receptor precursor gene *fptA* (2.5-fold).

Effect of NE on production of *P. aeruginosa* PA14 virulence factors

The levels of five virulence factors—pyocyanin, elastase, rhamnolipid, PQS, and pyoverdine—were determined in PA14 cultures exposed to 50 and 500 μM of NE. The

production of pyocyanin increased by 2.1±0.2-fold and 3.9±0.2-fold after 16 and 24 h of exposure to 50 μM NE (Fig. 2a) but did not change significantly at earlier time points. Similarly, exposure to 500 μM NE increased pyocyanin 3.2±0.9-fold, 4.9±0.5-fold, and 6.4±0.4-fold at 12, 16, and 24, respectively (Fig. 2a). NE exposure increased elastase production by 3.4±0.3-fold at 16 h and by 6.2±1.4 fold at 24 h upon exposure to 500 μM NE; however, elastase levels did not increase with 50 μM NE (Fig. 2b). The levels of PQS increased by 9.7±0.5-fold after 24-h exposure to 500 μM NE but not with 50 μM NE (Fig. 2c). Rhamnolipid levels were only marginally increased upon exposure to both 50 and 500 μM NE (1.7±0.1/1.8±0.1-fold at 16 h and 1.2±0.4/1.3±0.4-fold at 24 h; Fig. 2d). The levels of pyoverdine were not altered with either concentration of NE for up to 24 h (not shown).

We investigated the effect of increased virulence factor levels on attachment and invasion of the HCT-8 human enterocyte cell line. The extent of PA14 adhesion to HCT-8 cells in the presence of 50 and 500 μM NE increased by 1.6±0.3-fold and 2.9±0.4-fold, respectively (Fig. 3a). Similarly, invasion of HCT-8 cells was also increased by 1.5±0.2-fold and 4.2±0.5-fold upon exposure to 50 and 500 μM NE. Together with the increase in PA14 virulence factor levels, our data strongly indicate increased PA14 virulence and infectivity upon exposure to NE (especially at 500 μM NE).

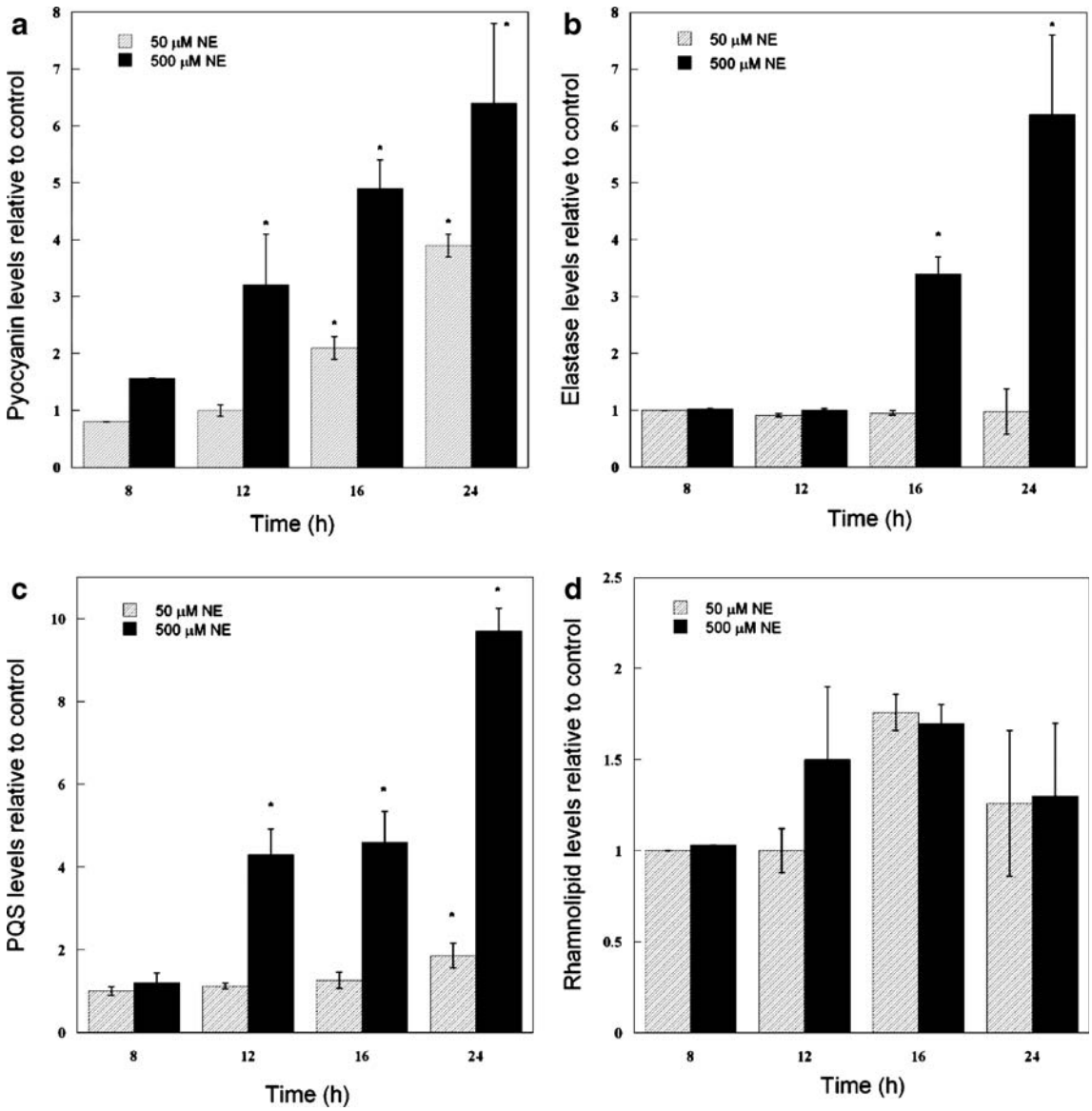

Fig. 2 Virulence factor production in the presence of NE. Changes in the levels of **a** pyocyanin, **b** elastase, **c** PQS, and **d** rhamnolipid in *P. aeruginosa* PA14 in the presence of 50 and 500 μM NE. Virulence factors were measured in triplicate after 8, 12, 16, and 24 h of exposure to NE and normalized to the cell density of the culture. Data shown are from three independent experiments. *Error bars* indicate mean fold change ± one standard deviation. *Asterisks*, indicate statistical significance at $p<0.05$ for NE-treated cultures relative to untreated control

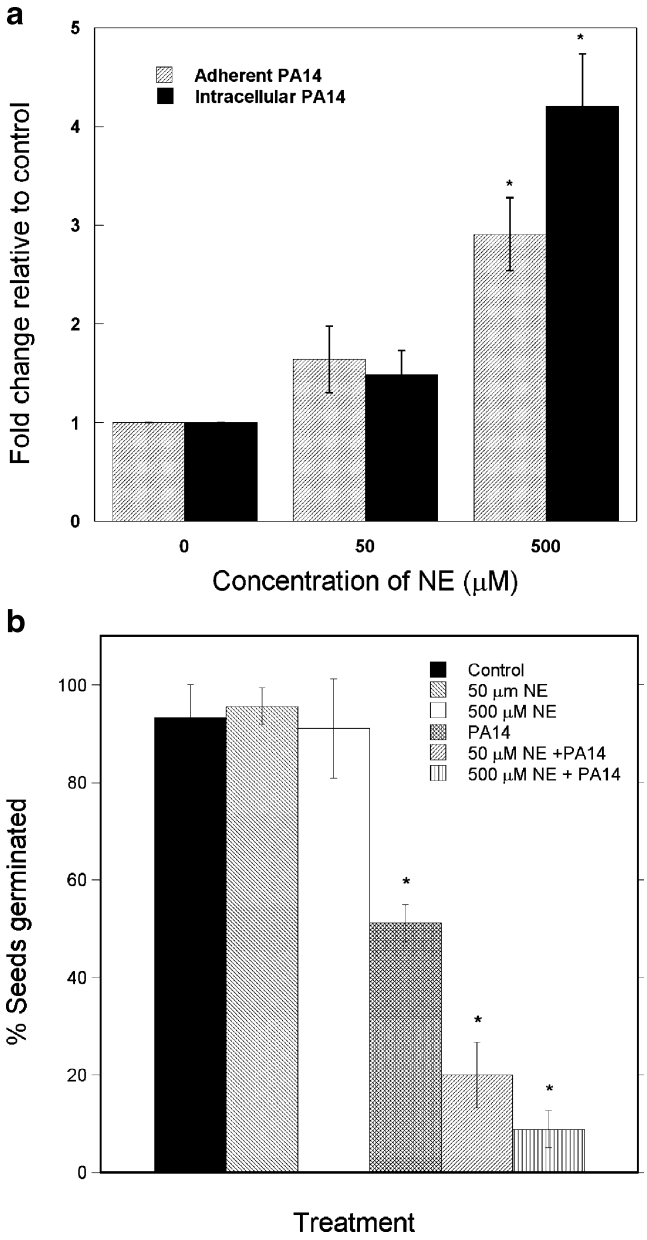

Fig. 3 Epithelial cell attachment and invasion and barley seed infection in the presence of NE. Relative changes in *P. aeruginosa* PA14 **a** attachment and invasion to HCT-8 cells and **b** barley seed germination, after exposure to 50 or 500 μM NE. For the attachment and invasion assays, cell counts (mean ± one standard deviation) are from duplicate LB agar plates and generated from nine HCT-8 cell culture wells and three independent experiments. *Asterisks* indicate statistical significance determined using a Student *t* test at $p<0.05$. For the barley seed germination assay, the percentage of barley seeds germinated was calculated. Data are the average of three independent experiments, and one standard deviation is shown. *Asterisks* indicate statistical significance determined using a Student *t* test at $p<0.001$

Since PA14 can infect both plant and animal hosts (He et al. 2004), we also investigated the effect of increased virulence factor levels on infection using a barley seed infection model (Attila et al. 2008a). Germination of barley seeds in the presence of PA14 was reduced to 51±4% ($p<0.005$) of the untreated control which is in good agreement with our prior work (Attila et al. 2008a). The addition of 50 and 500 μM NE further reduced germination to 20±7% and 9±4% of the control ($p<0.01$; Fig. 3b); hence, 50 and 500 μM NE increased *P. aeruginosa* virulence by 2.6±0.9-fold and 6±3-fold, respectively. Nearly 100% germination of barley seeds was observed in negative controls and seeds treated only with 50 or 500 μM NE (Fig. 3b). These results further confirm that exposure to NE increased PA14 virulence.

Effect of NE on *P. aeruginosa* PA14 swimming and swarming motility

Since motility is directly related to infection (Arora et al. 2005), we investigated the effect of NE PA14 swimming and swarming motility. The swimming motility of PA14 in serum-free RPMI with 0.5% agar supplemented with 10 μM $FeCl_3$ (i.e., the same base medium used for microarray and virulence factor experiments) increased in the presence of NE in a concentration-dependent manner (Fig. 4). Swimming motility was increased by 30% and 60% upon exposure to 50 and 500 μM NE. Interestingly, an extracellular product zone was detected in control and 50-μM NE plates outside the swimming motility halo but was absent in the 500-μM plates. The constituent of this clear zone was

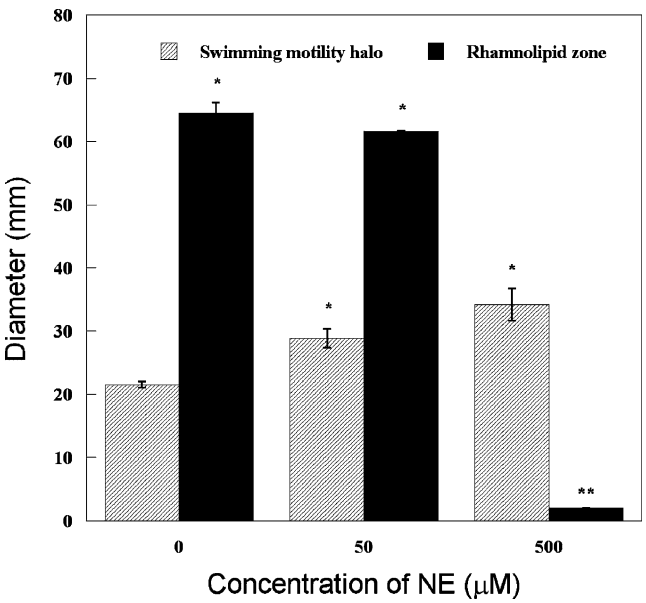

Fig. 4 Changes in *P. aeruginosa* PA14 swimming motility upon exposure to NE. PA14 was spotted onto RPMI 0.3% motility agar medium supplemented with 10 μM $FeCl_3$ containing 50 or 500 μM of NE. The swimming halo diameter and the clear rhamnolipid zone diameter were measured after 24 h at 37°C. *Error bars* indicate mean halo diameter or rhamnolipid zone diameter±one standard deviation. Data shown are from three independent cultures. *: statistical significance at $p<0.05$ for NE-treated cultures relative to control. **: diameter of the rhamnolipid zone with 500 μM outside the motility halo was negligible and could not be accurately measured

abiotic as it did not grow on plates and was identified as rhamnolipid using methylene blue staining (Caiazza et al. 2005). The diameter of this secreted rhamnolipid zone was only slightly less than the control with 50 μM NE but was virtually abolished at 500 μM (Fig. 4), suggesting that NE suppressed PA14 rhamnolipid production when growing on agar surfaces.

Since rhamnolipids contribute to *P. aeruginosa* swarming (Caiazza et al. 2005), we also investigated the effect of NE on PA14 swarming motility. Figure 5a–c shows that exposure to 500 μM NE completely inhibited PA14 swarming motility; however, no effect was observed with 50 μM NE. Since an increase in intracellular iron levels has been shown to decrease rhamnolipid production (Deziel et al. 2003), we investigated whether the NE-mediated decrease in swarming motility is due to an increase in intracellular iron levels. Figure 5d shows that increasing iron concentration in the swarming agar from 10 to 100 μM completely inhibited swarming motility similar to that observed with 500 μM NE.

NE enhances activity of *las*, but not *rhl*, quorum sensing

We hypothesized that the effects of NE at a concentration of 500 μM are mediated primarily through the *las*, but not the *rhl*, QS system. Therefore, we determined the transcriptional activation of *lasR* and *rhlR* (i.e., the response regulators of the *las* and *rhl* QS systems) with NE using a promoter::*lacZ* reporter fusion (Pesci et al. 1997) during the mid- to late-exponential growth phase (i.e., after 4, 5.5, 6.5, and 7.5 h of growth in the presence of NE). PA14 with the *plasR::lacZ* reporter plasmid showed a 3.2±0.8-fold increase in β-galactosidase activity in the presence of 500 μM NE after 7.5 h (Fig. 6) but did not demonstrate an increase in the presence of 50 μM NE. Although detectable β-galactosidase was observed at earlier time points (4, 5.5, and 6.5 h), the changes in activity observed with NE were not statistically significant from the control. On the other hand, PA14 with the *prhlR::lacZ* reporter did not show any change in β-galactosidase activity in the presence of NE (50 and 500 μM) compared to the untreated control at any of the time points. These results suggested that NE increases the activity of *las*, but not the *rhl*, QS pathway.

Discussion

It has been proposed that *P. aeruginosa* in the GI tract lumen can respond to specific environmental cues (e.g., high concentrations of hormones) by expressing different virulence determinants and initiating infection (Alverdy et

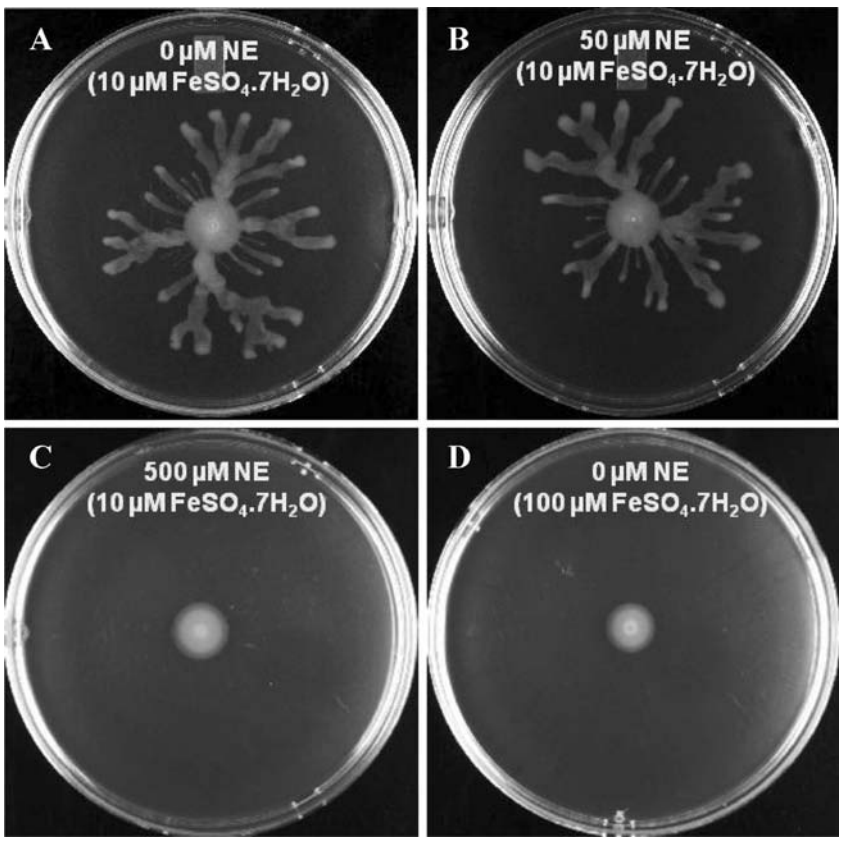

Fig. 5 Changes in swarming motility upon exposure to NE or excess iron. *P. aeruginosa* PA14 was spotted onto 0.5% BM2 motility agar containing 10 μM $FeSO_4$ and **a** 0 μM, **b** 50 μM, or **c** 500 μM NE. **d** Instead of NE, excess $FeSO_4$ was added to a final concentration of 100 μM. Representative images from three independent experiments are shown

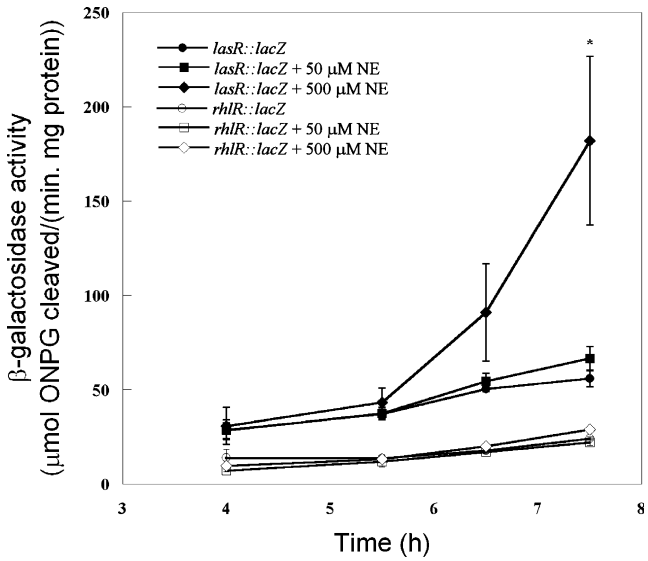

Fig. 6 Effect of NE on quorum-sensing pathways. Changes in *lasR* and *rhlR* expression were measured using the β-galactosidase activity assay. PA14 cultures containing plasmids with *lasR::lacZ* and *rhlR::lacZ* transcriptional fusions, respectively, were grown with 50 and 500 μM NE. The β-galactosidase activity in 1-mL culture aliquots was determined and normalized to the cell density. The β-galactosidase activity of PA14 cultures observed in plasmids with only *lacZ* (i.e., no *lasR* or *rhlR* fusion) was negligible. Data shown are from three independent experiments and represent mean β-galactosidase activity ± one standard deviation. *Asterisk* indicates statistical significance determined using a Student *t* test at $p<0.05$

al. 2000b). This hypothesis is supported by the fact that neuroendocrine hormones play an important role in gut-derived sepsis (Yang et al. 2000) and enter the circulation system (Aneman et al. 1996) and the lumen (Ahlman et al. 1981). In addition, intestinal levels of tyrosine hydroxylase, the rate-limiting enzyme in NE biosynthesis, have been shown to increase 2 h after the onset of sepsis and have been speculated to contribute to the increase in gut-derived NE during sepsis (Zhou et al. 2004). Although plasma levels of circulating catecholamines are in the nanomolar range during sepsis (Aneman et al. 1996), it should be noted that such plasma levels reflect spillover from tissue sites located throughout the body and do not accurately reflect the local concentration at any particular tissue site. For example, Kopin et al. (1984) have estimated that the concentration of NE at the receptors of vascular neuro-effector junctions (i.e., nerve endings that end in the vicinity of endothelial cells in the artery) during nonseptic conditions is at least threefold higher than that detected in the plasma. In fact, the plasma concentrations of hormones may underestimate the local concentration of NE in the GI tract by orders of magnitude (Leinhardt et al. 1993) as the mesenteric organs contribute to approximately half of the NE produced in the body (Aneman et al. 1995). Therefore, although the concentrations of NE (50 and 500 μM) used in this study do not reflect reported actual plasma concentrations, they are representative of the concentration range reported in the GI tract during normal and catabolic stress (Alverdy et al. 2000b; Eldrup and Richter 2000) and are consistent with concentrations used in previous studies (Cogan et al. 2007; Ernst et al. 1999; Lyte and Bailey 1997; Rasko et al. 2008; Vlisidou et al. 2004).

The increase in PA14 WT growth rate with 50 and 500 μM NE is consistent with in vitro studies showing that NE stimulates the growth of several bacterial pathogens, including *E. coli* and *P. aeruginosa* (Freestone et al. 1999; Lyte and Ernst 1992), in serum-based (i.e., transferrin-containing) nutritionally minimal growth media. It has been proposed that NE enhances pathogen growth by providing access to iron needed for growth (Freestone et al. 2000) through production of siderophores (Cox 1986). Although pyocyanin synthesis genes are upregulated on exposure to 500 μM NE, interestingly, genes involved in the production of other major *P. aeruginosa* siderophores such as pyoverdine and pyochelin (Lamont et al. 2002) were down-regulated upon NE exposure. Since several of these genes are regulated by the transcription factor Fur and repressed under conditions of iron abundance (Vasil and Ochsner 1999), our data suggest that PA14 preferentially utilizes pyocyanin for iron acquisition from a minimal-nutrient environment (Cox 1986) such as that seen in the GI tract, and the increase in iron levels leads to downregulation of other siderophore genes.

The increase in the production of the *P. aeruginosa* virulence factors such as pyocyanin and elastase suggests that the pathogen may opportunistically utilize host hormones to facilitate infection. Our data suggest pyocyanin may play a dual role in *P. aeruginosa* infections. Apart from stimulating PA14 growth through rapid acquisition of iron in minimal environments, pyocyanin is also likely involved in virulence as our data show pyocyanin levels increasing even after 24 h of exposure to NE (i.e., when the pathogen is no longer growing). The growth-independent production of pyocyanin likely increases the susceptibility of host cells to infection as pyocyanin has also been shown to cause oxidative stress in epithelial cells (Long et al. 2008) and apoptosis in neutrophils (Usher et al. 2002). Similarly, the increase in the levels of elastase, which is involved in degradation of elastin and collagen in host tissues during infection (Van Delden and Iglewski 1998), and PQS, which inhibits human T cell proliferation and acts as an immune modulator (Diggle et al. 2006; Hooi et al. 2004), also reinforces the idea that the NE-mediated increase in virulence factor levels could contribute to *P. aeruginosa* infections. The concordance between virulence factor production and attachment/invasion of epithelial cells supports the idea that PA14 could utilize NE during colonization and infection of the GI tract. The increase in

infection with the barley seed germination assay, a valid model for *P. aeruginosa* PA14 virulence as this strain contains a common set of virulence genes to elicit soft rot disease in lettuce and *Arabidopsis* plants, as well as in mice (Rahme et al. 2000), further strengthens the hypothesis that high concentrations of NE increase PA14 virulence.

Since NE is produced in situ in the GI tract (Aneman et al. 1996; Lyte 2004; Lyte and Bailey 1997), it is likely that pathogens in the GI tract encounter either low luminal concentrations of NE under normal conditions or short-lived bursts of higher NE concentrations during acute stress. The observation that sustained exposure (>8 h) to 500 μM, but not 50 μM, of NE is needed to cause an increase in pyocyanin, elastase, and PQS levels is significant in the context of gut-derived *P. aeruginosa* infections as it suggests that acute exposure to hormones is not likely to lead to *P. aeruginosa* infection.

The fact that NE significantly increased the levels of only pyocyanin (6.4-fold), elastase (6.2-fold), and PQS (9.7-fold), but not rhamnolipids (30%) and pyoverdine (unchanged), in suspension cultures at 24 h, argues that the increase in virulence factor levels is due to an NE-specific effect and not merely due to the increased cell density upon NE exposure. Four lines of evidence suggest that the *las* QS pathway is involved in mediating the effects of NE in PA14. First, the increase in expression of *lasA* and *lasB*, the upregulation of *lasR* promoter activity, and increased production of elastase strongly suggest the involvement of the *las* QS system. Second, the increase in the expression of other *las* QS-controlled *P. aeruginosa* virulence factor genes (phospholipase C) and in the expression of type II secretion genes (*xcpRSTUVXZ*; Filloux 2004) that is involved in secreting elastase and phospholipase and is itself controlled by *las* QS (Filloux 2004) is also indicative of the *las* QS being activated upon exposure to NE. Third, the twofold increase in the expression of the global response regulator *gacA*, which positively regulates *lasR* and the production of extracellular virulence factors such as pyocyanin, cyanide, and exoenzyme lipase (Reimmann et al. 1997), in the presence of 500 μM NE, suggests that NE may be acting through *gacA* to increase the *las* QS activity. Fourth, the increase in the levels of PQS also suggests increased *las* QS activity, as the expression of *mvfR* (upregulated threefold in our study), the regulator of PQS production, has been shown to be upregulated by *lasR* but downregulated by *rhlR* (Wade et al. 2005). The activation of *P. aeruginosa* QS by host hormones is also consistent with other studies showing that eukaryotic hormones such as epinephrine can influence *E. coli* O157:H7 quorum sensing (Kendall et al. 2007).

It was surprising to note that, while rhamnolipid levels were slightly increased or unchanged (16- and 24-h data points, respectively, in Fig. 2d) upon exposure to 500 μM NE in suspension cultures, they were significantly decreased on motility agar plates after 24 h. It is possible that the effect of NE on rhamnolipid production depends on the culture format in which PA14 is grown (i.e., in suspension or on semisolid surfaces). Overhage et al. (2008) recently reported that ~7.5% of the *P. aeruginosa* PAO1 genome, including genes involved in the type III secretion system, extracellular proteases, and siderophore synthesis, are differentially expressed under swarming conditions compared to suspension cells or biofilms. Since colonization on a semisolid surface closely mimics the in vivo scenario where a mucus layer covers epithelial cells, it is possible that the NE-mediated decrease in rhamnolipid production is significant in the context of PA14 virulence in vivo. Since colonization of epithelial cells is necessary for the infection process, the decrease in swarming motility observed with 500 μM NE could contribute, in part, to increased colonization and subsequent invasion. Further work is required to completely understand differences between *P. aeruginosa* pathogenesis in surface-associated and planktonic cultures.

In summary, we have shown that NE at a concentration of 500 μM, but not 50 μM, increases *P. aeruginosa* PA14 growth, motility, attachment, and virulence, all of which are integral to infection. Our data also show that the actions of NE are mediated primarily through the *las*, and not the *rhl* QS system. We propose that *P. aeruginosa* can utilize NE for colonization of the GI tract to initiate infection that eventually leads to gut-derived sepsis.

Note added in proof: Recent results obtained after submitting this manuscript show that the increase in *las* QS in the presence of norepinephrine can be replicated by the addition of iron alone (i.e., similar to the swarming motility results shown in Figure 5d), in that addition of 250 μM iron stimulates *lasR* transcription by 11-fold just as 500 μM norepinephrine stimulates *lasR* transcription by 3.2-fold. Therefore, the effects on NE on virulence phenotypes seem to be mediated, in part, through increased intracellular iron availability.

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