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The Biofilm-Specific Antibiotic Resistance Gene *ndvB* Is Important for Expression of Ethanol Oxidation Genes in *Pseudomonas aeruginosa* Biofilms

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Bacteria growing in biofilms are responsible for a large number of persistent infections and are often more resistant to antibiotics than are free-floating bacteria. In a previous study, we identified a *Pseudomonas aeruginosa* gene, *ndvB*, which is important for the formation of periplasmic glucans. We established that these glucans function in biofilm-specific antibiotic resistance by sequestering antibiotic molecules away from their cellular targets. In this study, we investigate another function of *ndvB* in biofilm-specific antibiotic resistance. DNA microarray analysis identified 24 genes that were responsive to the presence of *ndvB*. A subset of 20 genes, including 8 ethanol oxidation genes (*ercS′*, *erbR*, *exaA*, *exaB*, *eraR*, *pqqB*, *pqqC*, and *pqqE*), was highly expressed in wild-type biofilm cells but not in Δ*ndvB* biofilms, while 4 genes displayed the reciprocal expression pattern. Using quantitative real-time PCR, we confirmed the *ndvB*-dependent expression of the ethanol oxidation genes and additionally demonstrated that these genes were more highly expressed in biofilms than in planktonic cultures. Expression of *erbR* in Δ*ndvB* biofilms was restored after the treatment of the biofilm with periplasmic extracts derived from wild-type biofilm cells. Inactivation of ethanol oxidation genes increased the sensitivity of biofilms to tobramycin. Together, these results reveal that *ndvB* affects the expression of multiple genes in biofilms and that ethanol oxidation genes are linked to biofilm-specific antibiotic resistance.

Biofilms are the leading cause of hospital-acquired, implant-based infections and the basis of many persistent diseases, such as otitis media, periodontitis, and the chronic *Pseudomonas aeruginosa* lung infections that afflict patients with cystic fibrosis (CF) (4). The persistence of these infections is primarily attributed to the recalcitrance of biofilms to the immune system and antimicrobial agents. Bacteria growing in biofilms often show increased resistance to antibiotics (e.g., 10 to 100 times) compared to free-floating (planktonic) bacteria (20, 32). It is still not fully understood how biofilm cells become more resistant to antibiotics. Since the genetic makeup of the cells in the biofilm has not been altered, the increased resistance likely involves the altered expression of specific genes in the biofilm. It is well documented that the gene expression profile of biofilm cells is markedly different from that of planktonic cells (28, 34). Thus, a subset of these genes likely functions to protect biofilm cells from antibiotics.

We have identified several *P. aeruginosa* genes that contribute to biofilm-specific antibiotic resistance by screening for mutants with increased antibiotic sensitivity when growing in biofilms (21, 35, 36). One of these genes, *ndvB*, encodes a glucosyltransferase involved in the formation of cyclic glucans (21). The glucans are cyclic polymers of 12 to 15 β-(1→3)-linked glucose molecules with phosphoglycerol substitutions (27). Inactivation of *ndvB* blocked glucan production but did not affect growth, the kinetics of biofilm formation, or the architecture of the biofilms (21). However, biofilms of *ndvB* mutants exhibited increased sensitivity to the aminoglycosides tobramycin and gentamicin and the fluoroquinolone ciprofloxacin (21, 27). We and others have shown that antibiotics can physically interact with glucan-enriched periplasmic lysates and purified glucan preparations (21, 27). Thus, we proposed that glucans confer resistance to antibiotics by sequestering these antibiotics in the periplasm and away from their cytoplasmic targets (21).

Cyclic glucans have been studied primarily in *Rhizobium* species, in which they have roles in hypo-osmotic adaptation and in plant infection (2). In *Bradyrhizobium japonicum* and *Sinorhizobium meliloti*, secreted glucans are important for the symbiotic relationship between these bacteria and their plant hosts (6, 7). *ndvB* mutants are unable to produce functional root nodules on plants, and addition of exogenous cyclic glucans can enhance nodule formation (8). Thus, it has been suggested that the glucans are involved in signaling between the bacteria and plants, although the mechanism has not been elucidated (2). These observations led us to propose that *ndvB*-derived glucans might function in signaling in *P. aeruginosa*. In this study, we explored this possibility by investigating gene expression differences between wild-type and Δ*ndvB* bacteria growing in biofilms.

MATERIALS AND METHODS

Bacterial strains, media, and reagents. All *P. aeruginosa* strains used in this study were derivatives of the PA14 wild-type strain and are listed in Table 1 (26). Transposon mutants were retrieved from the nonredundant PA14 transposon library (18). The Δ*ndvB* mutant has been reported elsewhere (21). Additional unmarked deletion mutants were constructed in PA14 by allelic exchange with pEX18Gm derivatives as previously described (16, 21). Genes were cloned into the pJB866 vector as previously

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TABLE 1 *P. aeruginosa* strains and plasmids used in this study

Strain or plasmid	Genotype or description[a]	Source
Strains		
PA14	*P. aeruginosa* burn wound isolate	26
TFM15	PA14 Δ*ndvB*	21
TFM215	PA14 Δ*exaA*	This study
TFM212	PA14 Δ*pqqC*	This study
TFM220	PA14 Δ*erbR*	This study
TFM217	PA14 Δ*ndvB* Δ*exaA*	This study
TFM216	PA14 Δ*ndvB* Δ*pqqC*	This study
TFM221	PA14 Δ*ndvB* Δ*erbR*	This study
exaA::*MrT7*	PA14_38860::*MAR2* × T7	18
exaC::*MrT7*	PA14_38840::*MAR2* × T7	18
pqqC::*MrT7*	PA14_38800::*MAR2* × T7	18
eraR::*MrT7*	PA14_38900::*MAR2* × T7	18
Plasmids		
pJB866	Expression vector containing the *m*-toluic acid inducible promoter P*m*; Tc^r	1
pJB866-*exaA*	pJB866 with the PA14 *exaA* gene inserted downstream from the P*m* promoter	This study
pJB866-*pqqC*	pJB866 with the PA14 *pqqC* gene inserted downstream from the P*m* promoter	This study
pJB866-*erbR*	pJB866 with the PA14 *erbR* gene inserted downstream from the P*m* promoter	This study

[a] Tc^r, tetracycline resistant.

described (36), with open reading frames inserted downstream from the P*m* promoter, which is capable of induction by *m*-toluic acid (1). Primers used for the construction of deletion mutants and pJB866 derivatives are listed in Table 2. Bacteria were grown at 37°C in rich medium (Luria-Bertani [LB]) or minimal medium. The minimal medium (M63-arginine) was M63 salts supplemented with arginine (0.4%) and $MgSO_4$ (1 mM) (25). M63-ethanol was M63 salts supplemented with ethanol (0.5% [vol/vol]) and $MgSO_4$ (1 mM). Tobramycin and ciprofloxacin were purchased from Research Production International (Mt. Prospect, IL) and MP Biomedicals (Solon, OH), respectively.

DNA microarray analysis. RNA was extracted from PA14 wild-type and Δ*ndvB* biofilms grown in M63-arginine liquid medium using the Kadouri drip-fed method (23). Briefly, biofilms were grown in the wells of uncoated polystyrene 6-well plates for 48 h, washed, and detached through vigorous scraping. The cells were resuspended in fresh medium and centrifuged at 12,000 × *g*. The supernatant was removed, and the wash step was repeated. For both the wild-type and Δ*ndvB* strains, three biological replicate samples were analyzed, each of which derived from two wells of biofilm-grown cells. RNA was purified using the PureLink Micro-to-Midi total RNA purification system (Invitrogen) according to the manufacturer's instructions. Cells were lysed with a 10-ml syringe and a 20-gauge needle. An on-column digestion was performed with DNase (Sigma), and RNA was eluted in RNase-free water (Invitrogen). RNA was analyzed for concentration and purity by an Agilent 2100 Bioanalyzer at the StemCore Microarray Facility (Ottawa Hospital Research Institute). cDNA synthesis, labeling, and hybridization to *P. aeruginosa* PAO1 GeneChips were performed at the StemCore Microarray Facility.

Quality assessment and analysis of microarray data were performed using an open-source R programming environment (5) in conjunction with Bioconductor software (10). Background adjustments, normalization, and selection of differentially expressed genes were obtained by robust microarray analysis (RMA) and MicroArray Suite 5.0 (MAS5.0) procedures. The overlapping genes (identified in both the RMA and MAS5.0 procedures) were selected for further study. *t* tests were used to identify genes that showed significant changes in transcript levels ($P \leq 0.001$).

qPCR analysis. RNA was extracted from planktonic cultures, drip-fed biofilms, or colony biofilms grown at 37°C. Planktonic cultures were grown in LB to early stationary phase or in M63-arginine to exponential phase. Kadouri drip-fed biofilms were grown as described in the microarray experiment. Colony biofilms on M63-arginine agar plates were prepared as previously described (36), with incubation for 24 h at 37°C followed by 16 h at room temperature. RNA extraction and cDNA synthesis were performed as previously described (36). cDNA was quantified using SYBR green detection of PCR products with the MyiQ single-color detection system (Bio-Rad). Each 20-μl quantitative real-time PCR (qPCR) mixture contained 2 μl cDNA (~1.2 μg), 10 μl SYBR green PCR master mix (Applied BioSystems), and 100 pmol of each primer. The following thermal cycler conditions were used: 90 s at 95°C, followed by 45 cycles of 60 s at 95°C, 30 s at 56°C, and 30 s at 72°C. qPCR primers are listed in Table 2. RNAs isolated from at least two independent cell cultures were each tested in triplicate qPCRs, with expression of *rpoD* used as a reference standard. Statistical significance was determined by Student's *t* tests.

Planktonic growth in ethanol. To examine growth of bacteria using ethanol as the sole carbon source, overnight cultures grown in M63-arginine were diluted in 50 ml of M63 containing 0.5% ethanol to equivalent optical densities at 600 nm. For growth in M63-arginine or LB broth, overnight LB cultures were diluted into the appropriate medium. Cultures were incubated with shaking at 37°C, and planktonic growth was monitored by absorbance readings at 600 nm. At least two biological replicates were performed for each condition.

Antibiotic resistance assays. Minimum bactericidal concentrations (MBCs) were determined for biofilm (MBC-B) and planktonic (MBC-P) cultures as previously described (21, 36). Briefly, overnight cultures of bacteria were diluted (1:50) in M63-arginine in 96-well microtiter plates. For MBC-B assays, biofilms were allowed to form for 24 h, planktonic cells were removed, and the biofilms were exposed to serial dilutions of antibiotics. For MBC-P assays, antibiotics were added at the same time that the plates were inoculated. After 24 h of antibiotic exposure, bacterial survival was determined by spotting a small amount (ca. 3 μl) of culture on LB agar plates immediately after antibiotic treatment (MBC-P) or following a 24-h recovery period in which surviving cells can detach from biofilms into antibiotic-free growth medium (MBC-B). For MBC-B assays on ethanol minimal medium, M63 containing 0.5% ethanol as the carbon source was used.

For another measure of planktonic antibiotic resistance, MICs were determined in LB broth using the 2-fold broth dilution method (3). For strains carrying pJB866 derivatives, MICs were determined in LB or M63-arginine containing 2 mM *m*-toluic acid to induce expression of cloned genes.

Signaling experiment. Periplasmic extracts were isolated from 750-ml PA14 planktonic cultures grown in M63-arginine, as previously described (21). The cells were pelleted, and the periplasmic contents were extracted with 70% ethanol at 70°C for 30 min. The insoluble fraction was removed by centrifugation, and the supernatant was precipitated by adding NaCl to 0.1 M and 10 volumes of 100% ethanol. After centrifugation for 30 min at 7,700 × *g*, the pellet was dried and suspended in 10 ml water. The presence of glucans in the periplasmic extracts was confirmed using the anthrone-sulfuric acid method of carbohydrate quantification (19, 21). Glucans were purified by gel filtration chromatography of periplasmic extracts with Sephadex G-75 resin, as previously described (21). The glucan-containing fractions were dried by Speed-Vac evaporation, and the pellets were suspended in water.

For the signaling experiment, the periplasmic extracts and purified glucans were added to wild-type and Δ*ndvB* static biofilms that were pregrown for 48 h in 3 ml M63-arginine (with the medium refreshed at 24 h). Wild-type periplasmic extract (65 μg of anthrone-positive material), Δ*ndvB* periplasmic extract (volume equivalent to that of the wild-type extract), purified glucans (65 μg of anthrone-positive material), or water was added to the biofilms. The volumes of the treatments were standardized to 144 μl with water. The biofilms were incubated for 8 h, RNA was

TABLE 2 Oligonucleotides used in this study

Primer	Sequence (5′ to 3′)[a]	Function
exaAf1	CCTA**GAATTC**GCGCAGTTCTGGTTGTAGGC	Deletion of *exaA*
exaAr2	AACC**GAGCTC**CGTGGATCAGCAGGACCTTG	Deletion of *exaA*
exaAf3	AATT**GAGCTC**TCCGGATCAAGCGCATGTACG	Deletion of *exaA*
exaAr4	ATTG**AAGCTT**GAAGTTGCCGAAGCGCACCT	Deletion of *exaA*
pqqCf1	AACT**GAGCTC**TAACTCGACCTGCCGGAATC	Deletion of *pqqC*
pqqCr2	AACT**GGTACC**GTACATCGCGACATGGAAC	Deletion of *pqqC*
pqqCf3	AACT**GGTACC**GACAGTTGGCCGCGCCACTA	Deletion of *pqqC*
pqqCr4	ATCC**AAGCTT**CCGCTCGATGTTGTCGATG	Deletion of *pqqC*
agmR-StrEcoR Ib	GTCA**GAATTC**GCAACAGGCGGTAGAGATGG	Deletion of *erbR*
agmR-StrXbaI-R	CAAC**TCTAGA**CGATGACATTGTGGATGG	Deletion of *erbR*
agmR-XhaI-F	CAAC**TCTAGA**AAGCTCAAGGTACACAACC	Deletion of *erbR*
agmR-HindIII Ib	GGCT**AAGCTT**GCTGATCGTCGACTATCAC	Deletion of *erbR*
ndvB JB-F	GTTG**GAGCTC**ATGTCTTCACGCAAGATCGG	Cloning *ndvB* into pJB866
ndvB JB-R	GTTG**AAGCTT**ACCTCAACCGCCGATCTGCTC	Cloning *ndvB* into pJB866
agmR JB-F2	GTTC**AAGCTT**CATGTACAAGATCCTGATCGC	Cloning *erbR* into pJB866
agmR JB-R2	GTTC**CTCGAG**AGCATCGCCAGGACAGTGAG	Cloning *erbR* into pJB866
exaA JB-F	ATCA**GAGCTC**ATGACAACAAGAACCTCACC	Cloning *exaA* into pJB866
exaA JB-F	GTTC**AAGCTT**ATCGGGATACTCCGCAGGCG	Cloning *exaA* into pJB866
pqqC JB-F	ATCA**GACTCA**TGAGCCGTGCCGCCATGGA	Cloning *pqqC* into pJB866
pqqC JB-R	GTTC**AAGCTT**ATCGGCACGCTGTCGAGCGA	Cloning *pqqC* into pJB866
rpoD-QF	CATCCGCATGATCAACGACA	*rpoD* qPCR primer
rpoD-QR	GATCGATATAGCCGCTGAGG	*rpoD* qPCR primer
ndvB-QF	GGCCTGAACATCTTCTTCACC	*ndvB* qPCR primer
ndvB-QR	GATCTTGCCGACCTTGAAGAC	*ndvB* qPCR primer
exaA-QF	AGACCAACACCATCATCGTC	*exaA* qPCR primer
exaA-QR	GGTGTGCTGGTAGAACCACT	*exaA* qPCR primer
exaB-QF	CTCCGCCTACAACCAGAACT	*exaB* qPCR primer
exaB-QR	CCTTCCTGGCTGATGAAGTC	*exaB* qPCR primer
exaC-QF	GGTCAAGGGCCAGTACTTCA	*exaC* qPCR primer
exaC-QR	GCGATCTTCAGCAGGATGTT	*exaC* qPCR primer
exaE-QF	AAATTCCTTCGCTGGTCATC	*eraR* qPCR primer
exaE-QR	AAGAACAGCACACGCAACTG	*eraR* qPCR primer
agmR-QF	CATTCCCGTGGTTATCGTCT	*erbR* qPCR primer
agmR-QR	AGGTAGACGTTGCCGTTGAG	*erbR* qPCR primer
pqqB-QF	GACGAGATGCTGGTCTGC	*pqqB* qPCR primer
pqqB-QR	ATTGGTGTTGTTGATGTGGA	*pqqB* qPCR primer
pqqC-QF	CGATAGTGCTCCAGGGTGAT	*pqqC* qPCR primer
pqqC-QR	GTCGACGCCTACGTCAACTT	*pqqC* qPCR primer
pqqE-QF	GGCACAACATCGACAACATC	*pqqE* qPCR primer
pqqE-QR	GTAGTAGTCGGGGGTGACGA	*pqqE* qPCR primer
ercS′-QF	CGAACTGAAGGCCAGCAACC	*ercS′* qPCR primer
ercS′-QR	AGGACGAAGGCGTCGGAGAT	*ercS′* qPCR primer

[a] Locations of restriction sites are underlined and in bold.

isolated from the biofilms, and *erbR* expression was assayed by qPCR. The results represent two or three biological replicate samples, each tested in triplicate.

Microarray data accession number. The microarray data have been deposited at the Gene Expression Omnibus (GEO) database (accession no. GSE32032).

RESULTS

***ndvB* inactivation results in differential transcription of 24 genes in biofilms.** To further investigate the function of *P. aeruginosa* glucans in biofilm-specific antibiotic resistance, we compared the global gene expression profiles of wild-type and Δ*ndvB* biofilms. The biofilms were grown in M63-arginine for 48 h in a Kadouri drip-fed system. Microarray analysis of biofilm RNA led to the identification of 24 genes with differential expression in the Δ*ndvB* biofilms (Table 2). Twenty of the genes were expressed preferentially in wild-type biofilms, and four of the genes were more highly expressed in Δ*ndvB* biofilms.

While most of the *ndvB*-responsive genes are predicted to encode proteins of unknown or unrelated functions, 8 of the genes are involved in ethanol oxidation (Table 3) (14, 22). These include the gene encoding a quinoprotein ethanol dehydrogenase (*exaA*) and genes involved in the synthesis of its cofactor pyrroloquinoline-quinone (PQQ) (*pqqBCE*). The PQQ-bound ethanol dehydrogenase is a periplasmic enzyme required for aerobic growth using ethanol as the sole source of carbon and energy (14). Another *ndvB*-responsive gene, *exaB*, encodes a cytochrome required for electron transport during growth on ethanol (29). Transcription of these genes is hierarchically controlled by at least six transcriptional regulators (22), three of which showed reduced expression in the Δ*ndvB*

TABLE 3 Genes differentially expressed in wild-type and Δ*ndvB* biofilms[a]

PA14 gene[b]	*P. aeruginosa* PAO1 orthologue	Fold change[c]	*P* value ($\times 10^{-5}$)	Gene name	Function[d]
PA14_03900	PA0299	−2.36	4.48	*spuC*	Putrescine-pyruvate aminotransferase
PA14_09320	PA4222	−2.76	8.57	*pchI*	ATP-binding component of an ABC transporter
PA14_11210	PA4072	2.19	7.48		Putative amino acid permease
PA14_24720	PA3044	2.76	1.07		Putative two-component sensor
PA14_29040	PA2715	2.10	1.04		Putative ferredoxin
PA14_29220	PA2700	2.43	1.41	*opdB*	Putative porin
PA14_36120	PA2210	2.99	1.33		Putative MFS transporter
PA14_38780	**PA1989**	**2.82**	**1.11**	***pqqE***	**PQQ biosynthesis protein E**
PA14_38800	**PA1987**	**2.11**	**1.09**	***pqqC***	**PQQ biosynthesis protein C**
PA14_38820	**PA1986**	**3.75**	**3.86**	***pqqB***	**PQQ biosynthesis protein B**
PA14_38850	**PA1983**	**2.95**	**2.51**	***exaB***	**Cytochrome c_{550}**
PA14_38860	**PA1982**	**3.75**	**5.67**	***exaA***	**Ethanol dehydrogenase**
PA14_38900	**PA1980**	**3.36**	**3.37**	***eraR***	**Response regulator (formerly *exaE*)**
PA14_38930	**PA1978**	**2.89**	**1.89**	***erbR***	**Response regulator (formerly *agmR*)**
PA14_38970	**PA1976**	**3.89**	**2.89**	***ercS′***	**Sensor kinase**
PA14_38990	PA1975	3.01	2.03		Hypothetical
PA14_51205	PA1015	2.10	6.51		Putative transcriptional regulator
PA14_52230	PA0931	2.13	2.98	*pirA*	Ferric enterobactin receptor
PA14_57275	PA4407	−2.30	5.14	*ftsZ*	Cell division protein
PA14_58890	PA4540	2.53	6.15		Hypothetical
PA14_64240	PA4857	5.10	4.73		MarC family protein
PA14_64680	PA4894	2.74	7.54		Hypothetical
PA14_70140	PA5312	−2.90	2.41	*aldH*	Putative aldehyde dehydrogenase
PA14_71250	PA5397	2.14	8.46		Hypothetical

[a] This list includes all genes differentially expressed by at least 2-fold in a DNA microarray analysis comparing wild-type and Δ*ndvB* biofilms ($P < 10^{-4}$).
[b] Genes involved in ethanol oxidation are indicated in bold. A gene coregulated with ethanol oxidation genes is underlined.
[c] Values indicate the fold change in gene expression between wild-type and Δ*ndvB* biofilms. Positive values indicate greater expression in wild-type biofilms. Negative values indicate greater expression in Δ*ndvB* biofilms.
[d] MFS, major facilitator superfamily.

biofilms (*eraR*, *erbR*, and *ercS′*). All of the ethanol oxidation genes discussed here, plus another uncharacterized gene identified in the microarray analysis (PA14_38990), are situated in the same genetic locus (Fig. 1). Since a large proportion of *ndvB*-responsive genes occupies this locus, we chose to investigate the genes further.

Expression of ethanol oxidation genes in biofilms involves NdvB and ErbR. The microarray experiment indicated that the expression of ethanol oxidation genes in *P. aeruginosa* biofilms requires *ndvB*. We used quantitative real-time PCR (qPCR) of RNA isolated from biofilms to validate the *ndvB*-dependent expression of the eight ethanol oxidation genes identified in the microarray analysis. As shown in Fig. 2, expression of each gene was reduced in Δ*ndvB* biofilms relative to that in wild-type biofilms, confirming the microarray results. In addition, the genes were more highly expressed in wild-type biofilms than in planktonic cells (Fig. 2). These data suggest that the ethanol oxidation genes

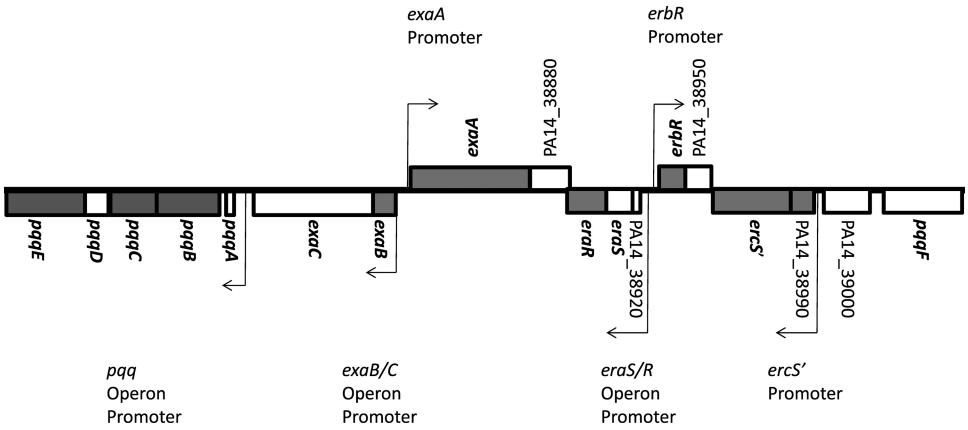

FIG 1 Chromosomal arrangement of ethanol oxidation genes in *P. aeruginosa* strain PA14. *ndvB*-responsive genes identified by microarray analysis are shown in gray. Genes previously reported to be involved in ethanol oxidation are in bold (14, 22). The confirmed promoters for several operons of ethanol oxidation genes are indicated by arrows (12, 13, 22).

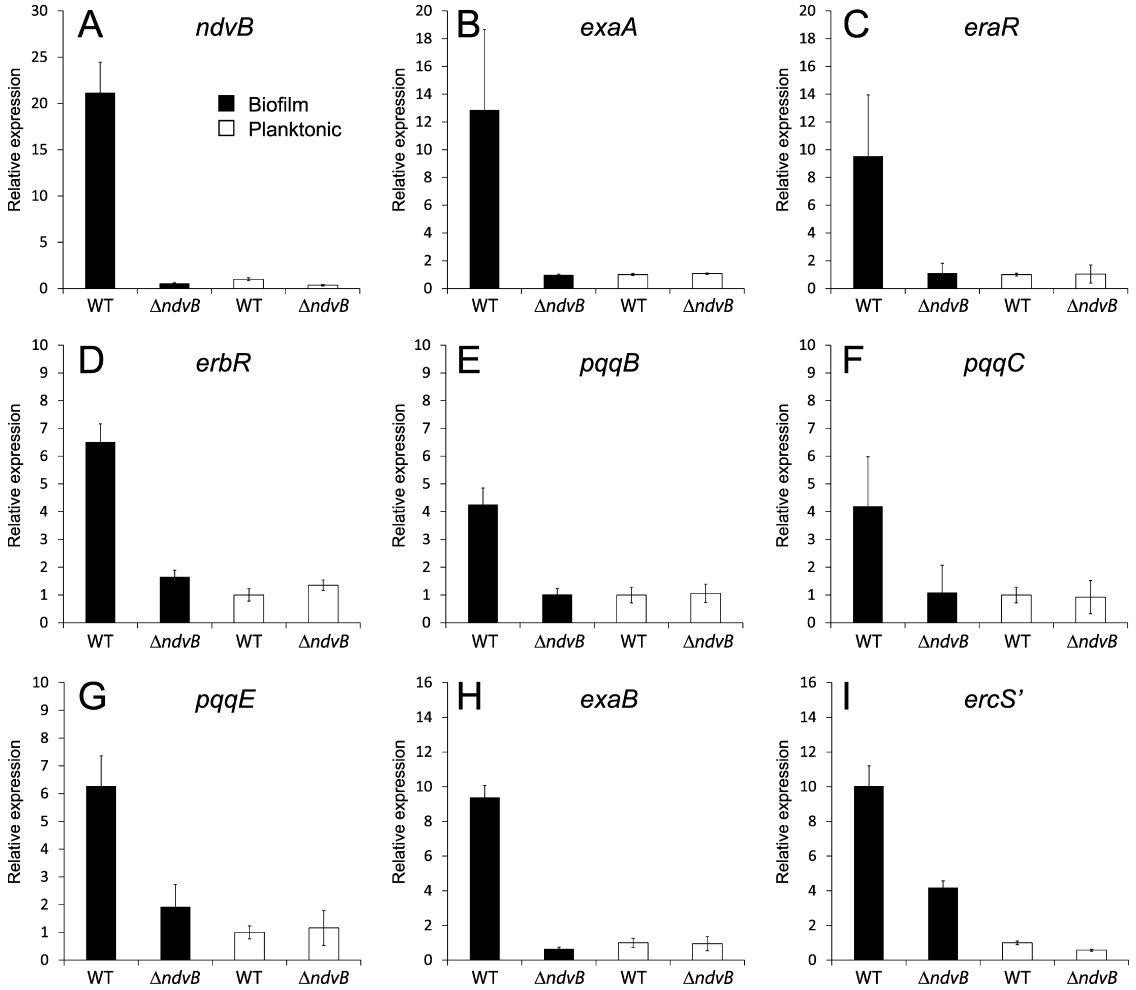

FIG 2 qPCR analysis of ethanol oxidation genes in biofilms and planktonic cultures of wild-type and Δ*ndvB* strains. RNA was extracted from drip-fed biofilms grown in M63-arginine and planktonic cultures grown in LB (A to G) or from colony biofilms and planktonic cultures that were both grown in M63-arginine (H and I). Gene expression of the wild type and the Δ*ndvB* mutant under each condition is given relative to that of the wild-type planktonic cultures. The error bars indicate standard deviations for two biological replicates tested in triplicate qPCRs. Tested genes include *ndvB* (A), *exaA* (B), *eraR* (C), *erbR* (D), *pqqB* (E), *pqqC* (F), *pqqE* (G), *exaB* (H), and *ercS′* (I). All of the genes showed a statistically significant ($P < 0.05$) increase in expression in wild-type biofilms compared to that in Δ*ndvB* biofilms. WT, wild type.

are induced in *P. aeruginosa* biofilms and that *ndvB* is required for this induction (21).

The genes encoding the *P. aeruginosa* ethanol oxidation system are controlled by a hierarchical signal transduction network (13, 22). Previous work with *P. aeruginosa* strain ATCC 17933 found that the response regulator ErbR (formerly AgmR) is a key member of this network (13). In the presence of ethanol, ErbR was shown to directly activate transcription of the *exaBC*, *eraSR*, and *pqqABCDE* operons and indirectly activate transcription of *exaA* (13). We suspected that the *ndvB*-dependent induction of the ethanol oxidation genes in biofilms also requires *erbR*. Therefore, we examined expression of three genes with different promoters (*exaA*, *exaB*, and *pqqB*) in wild-type and Δ*erbR* strains. Each gene was induced in wild-type biofilms relative to its expression in planktonic cells (Fig. 3), and gene expression was diminished in the Δ*erbR* biofilms, indicating that *erbR* is involved in the induction (Fig. 3). However, expression was not completely abolished in the Δ*erbR* biofilms, suggesting that other regulatory mechanisms contribute to the induction of these genes in biofilms. Overall, these results indicate that the induction of ethanol oxidation genes in *P. aeruginosa* biofilms involves both known (*erbR*) and novel (*ndvB*) regulatory elements.

Planktonic growth in ethanol is compromised in the Δ*ndvB* mutant. A potential consequence of the reduced expression of the ethanol oxidation genes in the Δ*ndvB* mutant is suboptimal growth on ethanol. We tested this possibility by comparing planktonic growth on ethanol minimal medium of the Δ*ndvB* mutant, the wild type, and three mutants lacking ethanol oxidation genes (Δ*exaA*, Δ*pqqC*, and Δ*erbR*). The Δ*ndvB* mutant had an intermediate growth rate that was substantially slower than that of the wild-type strain (Fig. 4A). In agreement with previous work, the Δ*exaA*, Δ*pqqC*, and Δ*erbR* mutants did not grow on ethanol (13, 14). The growth phenotypes were specific to the ethanol medium, since each of the strains exhibited wild-type growth rates on arginine minimal medium (Fig. 4B) and LB broth (Fig. 4C). The compromised planktonic growth of the *ndvB* mutant on ethanol is consistent with the regulatory link between *ndvB* and the ethanol oxidation genes.

Inactivation of ethanol oxidation genes increases the antibiotic sensitivity of biofilms. The biofilm-specific induction of eth-

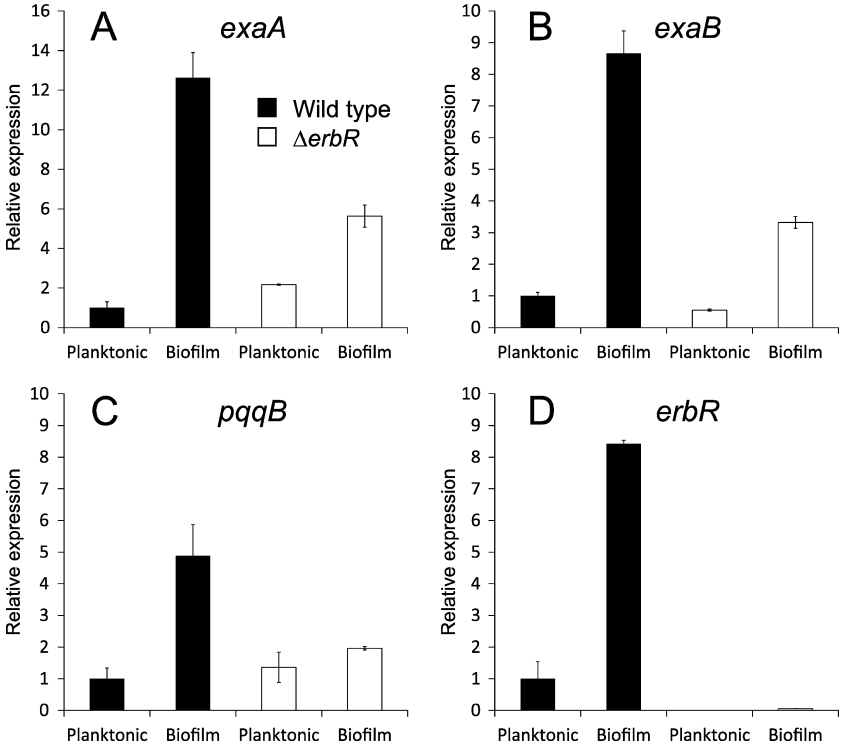

FIG 3 Induction of *exaA*, *exaB*, and *pqqB* in biofilms involves the response regulator ErbR. Colony biofilms and planktonic cultures of wild-type and Δ*erbR* strains were grown in M63-arginine. RNA was assayed by qPCR for expression of *exaA* (A), *exaB* (B), *pqqB* (C), and *erbR* (D). Gene expression is given relative to that of the wild-type planktonic cultures. For each condition, two biological replicate samples were tested in triplicate qPCRs.

anol oxidation genes was surprising given that the biofilms in these experiments were grown with medium lacking known inducers (i.e., ethanol and other alcohols) (12, 30). This observation suggested that the genes may have a function in biofilms that is unrelated to ethanol oxidation. Since *ndvB* is important for biofilm-specific antibiotic resistance (21), we tested whether the ethanol oxidation genes contribute to antibiotic resistance. We obtained transposon insertion mutants of 4 ethanol oxidation genes (*exaA*, *exaC*, *pqqC*, and *eraR*) from the PA14 transposon mutant library (18). We tested their antibiotic resistance phenotypes under both planktonic and biofilm growth conditions by determining the minimum bactericidal concentrations (MBCs) of ciprofloxacin, an antibiotic used to treat *P. aeruginosa* infections (11). We found that the mutants did not differ substantially from the wild-type strain in growth rate, biofilm formation ability, or planktonic antibiotic resistance (Table 4 and data not shown). However, compared to the wild-type strain, the mutants were more sensitive to ciprofloxacin when growing in biofilms (Table 4). These data suggested that ethanol oxidation genes contribute to the antibiotic resistance of biofilms.

To validate these results, we tested the antibiotic resistance phenotypes of unmarked deletions of *exaA*, *pqqC*, and *erbR*. We determined MBCs for planktonic and biofilm cells (Table 5) and MICs for planktonic cells (Table 6). Tobramycin sensitivity was observed in the deletion mutant biofilms, although ciprofloxacin sensitivity was not observed (Table 5). Consistent with the role of

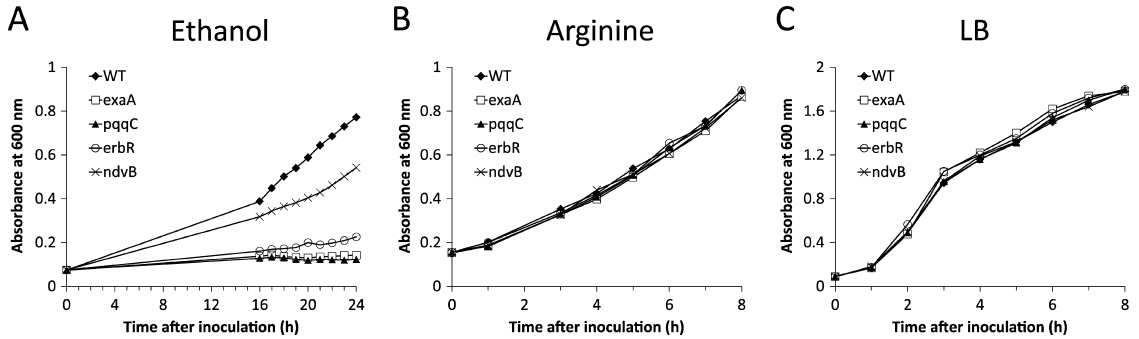

FIG 4 Inactivation of *ndvB* compromises planktonic growth on ethanol. The PA14 wild-type strain (WT), the Δ*ndvB* mutant, and three ethanol oxidation gene mutants (Δ*exaA*, Δ*pqqC*, and Δ*erbR*) were grown in M63 containing ethanol (A) or arginine (B) as the sole carbon source or in LB medium (C). Growth was monitored by measuring absorbance at 600 nm. The growth curves are representative of at least two replicate experiments. Statistically significant differences in growth between the wild-type and mutant strains ($P < 0.05$) were observed for ethanol but not for the other conditions.

TABLE 4 Minimum bactericidal concentrations of ciprofloxacin for PA14 transposon mutants[a]

Strain[b]	Ciprofloxacin MBC (μg/ml)	
	MBC-P	MBC-B
PA14	3	20
PA14 Δ*ndvB*	3	5
exaA::*MrT7*	3	10
exaC::*MrT7*	3	10
pqqC::*MrT7*	3	10
eraR::*MrT7*	3	10

[a] Minimum bactericidal concentrations were determined for planktonic (MBC-P) and biofilm (MBC-B) cultures grown in M63-arginine.
[b] Transposon mutants were retrieved from the nonredundant library of PA14 transposon insertion mutants (18).

these genes in biofilm-specific antibiotic resistance, mutants with double deletions (i.e., Δ*ndvB* plus deletion of one of the three genes listed above) displayed increased susceptibility to tobramycin in comparison to those with single deletions (Table 5). Furthermore, we performed an MBC assay comparing the resistance of Δ*ndvB* biofilms grown in ethanol minimal medium to that of the Δ*ndvB* biofilms grown in arginine minimal medium. We found that growth in ethanol resulted in a slight but reproducible increase in resistance to tobramycin, suggesting that activation of the ethanol oxidation genes partially restores antibiotic resistance.

To confirm the importance of these genes in antibiotic resistance, we tested whether induction of *ndvB*, *erbR*, *exaA*, and *pqqC* in the wild-type background increases planktonic antibiotic resistance. The genes were cloned into pJB866, a vector carrying the P*m* promoter, which can be induced by *m*-toluic acid (35, 36). We determined the MICs of PA14 carrying each vector under inducing conditions (2 mM *m*-toluic acid). Compared to the vector control, the vectors with cloned genes increased the tobramycin MIC of PA14 by 2-fold for *ndvB*, *exaA*, and *pqqC*, with no MIC change for *erbR*. The vectors also increased the gentamicin MIC by 2-fold (for *erbR*) or 4-fold (for *exaA* and *pqqC*). The *ndvB* vector increased the ciprofloxacin MIC of PA14 by 2-fold.

We also expressed *erbR*, *ndvB*, *exaA*, and *pqqC* in the respective deletion mutant strains. Expression of *erbR* in an Δ*erbR* strain resulted in an 8-fold increase in resistance to ciprofloxacin in an MIC assay. Expression of *ndvB* in an Δ*ndvB* mutant strain resulted in a 2-fold increase in resistance to ciprofloxacin. However, expression of *exaA* or *pqqC* had no effect on their respective deletion mutant strains. Thus, increased expression of several ethanol oxidation genes resulted in increased resistance to tobramycin, gentamicin, and ciprofloxacin, further indicating that these genes contribute to antibiotic resistance.

TABLE 5 Minimum bactericidal concentrations for PA14 deletion mutants

Strain	MBC (μg/ml)			
	Tobramycin		Ciprofloxacin	
	MBC-P	MBC-B	MBC-P	MBC-B
PA14	16	200	2	20
PA14 Δ*ndvB*	16	50	2	10
PA14 Δ*exaA*	8	50	1	20
PA14 Δ*pqqC*	16	100	2	20
PA14 Δ*erbR*	16	50	1	20
PA14 Δ*ndvB* Δ*exaA*	8	25	1	10
PA14 Δ*ndvB* Δ*pqqC*	16	25	1	10
PA14 Δ*ndvB* Δ*erbR*	16	25	0.5	20

TABLE 6 MICs for planktonic cultures of PA14 deletion mutants

Strain	MIC (μg/ml)	
	Tobramycin	Ciprofloxacin
PA14	2	1
PA14 Δ*ndvB*	2	1
PA14 Δ*exaA*	2	1
PA14 Δ*pqqC*	2	1
PA14 Δ*erbR*	2	1

Expression of *erbR* and *exaA* is restored in Δ*ndvB* biofilms after incubation with wild-type periplasmic extract. The *ndvB*-dependent induction of the ethanol oxidation genes in biofilms is likely an ethanol-independent mechanism, since the gene expression experiments were carried out in medium lacking ethanol. In *Rhizobium* species, NdvB-derived cyclic glucans are important for symbiotic interactions with plants, possibly by mediating signaling between bacteria and plants (2). We reasoned that a signaling mechanism involving glucans might be responsible for the gene expression changes we observed in *P. aeruginosa* biofilms. Since both NdvB-derived glucans and the ethanol dehydrogenase localize to the periplasm (14, 21), it seemed possible that a periplasmic signaling molecule present in wild-type biofilms might be absent in Δ*ndvB* biofilms. Thus, adding the signal in *trans* to Δ*ndvB* biofilms might restore expression of ethanol oxidation genes to wild-type levels.

We isolated periplasmic extracts from late-stationary-phase planktonic cultures of the wild-type strain, a condition that results in *ndvB* gene expression and glucan production (21). To determine whether a component of the extract might affect transcription of ethanol oxidation genes, we added wild-type periplasmic extract to preformed wild-type and Δ*ndvB* biofilms and incubated for 8 h. We assayed *erbR* and *exaA* expression and found that the wild-type periplasmic extract restored gene expression in the Δ*ndvB* biofilms to wild-type levels and also increased expression in the wild-type biofilms (Fig. 5A and B). In a control experiment, periplasmic extract isolated from Δ*ndvB* cultures did not restore wild-type expression of *erbR* (Fig. 5C).

These data suggest that a periplasmic molecule is capable of inducing *erbR* and *exaA* gene expression in the Δ*ndvB* mutant. We suspected that the signal might be the NdvB-derived glucans. Therefore, we purified glucans from PA14 periplasmic extracts by gel filtration (21). However, treatment of the Δ*ndvB* biofilms with purified glucans did not induce expression of *erbR* or *exaA* (Fig. 5A and B). Thus, the induction of ethanol oxidation genes appears to be modulated by an unidentified periplasmic molecule that is dependent on the presence of *ndvB*.

DISCUSSION

The increased antibiotic resistance of biofilms continues to confound treatment of bacterial infections (4, 15). We investigate *ndvB*, a gene which contributes to the increased antibiotic resistance of *P. aeruginosa* biofilms. *ndvB* encodes a glucosyltransferase required for the formation of cyclic periplasmic glucans (21, 27). We have previously shown that *ndvB*-derived glucans can interact

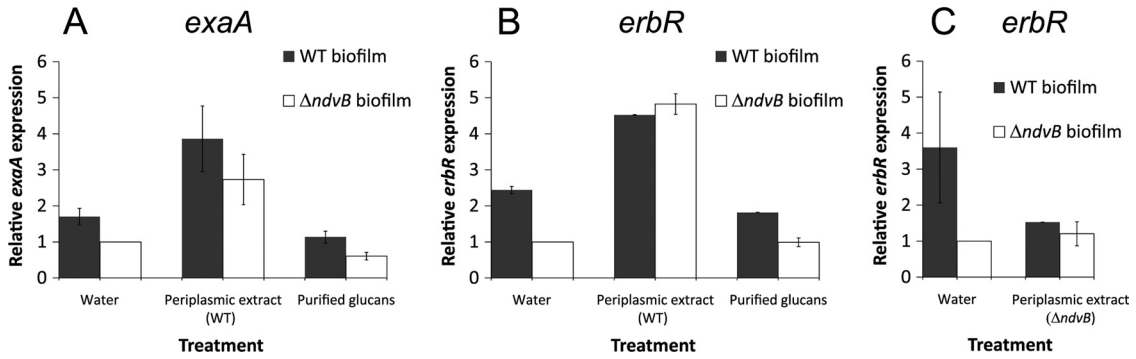

FIG 5 Expression of *erbR* and *exaA* in biofilms treated with periplasmic extract or purified glucans. Preformed wild-type and Δ*ndvB* biofilms were treated for 8 h with water (negative control), wild-type periplasmic extract, Δ*ndvB* periplasmic extract, or purified glucans. Expression of *exaA* (A) and *erbR* (B and C) was measured by qPCR and is given relative to that of the untreated Δ*ndvB* biofilms. Each panel shows the results of two or three biological replicates tested in triplicate qPCRs.

with antibiotics and, thus, sequester these compounds away from their cellular targets (21). In this study, we explore other functions of *P. aeruginosa* glucans by examining how global gene expression is affected by their presence or absence. DNA microarray and qPCR analyses revealed that several genes required for ethanol oxidation are responsive to the presence of *ndvB* in the genome. These genes have greater expression levels in biofilms than in planktonic cells, and their inactivation increased the antibiotic sensitivity of bacteria growing in biofilms. Additional genes were also identified as *ndvB* responsive in this study, but their role in antibiotic resistance is unknown.

The *P. aeruginosa* ethanol oxidation system has been studied only in planktonic cultures, and its function in biofilms is unexplored. We found that eight ethanol oxidation genes are induced in *P. aeruginosa* biofilms. The cause of the induction is unclear, since these genes are not expressed in planktonic cultures in the absence of known inducers (12, 30). One explanation is that ethanol is produced in wild-type, but not Δ*ndvB*, biofilms at levels sufficient to activate the ethanol oxidation genes. The most likely route of ethanol production in *P. aeruginosa* is as an anaerobic fermentation product. Steep oxygen gradients exist in laboratory biofilms exposed to air, and it is possible that a subpopulation of bacteria grows anaerobically (33). Nevertheless, ethanol production has not been demonstrated in *P. aeruginosa* cultures. In anaerobic conditions, *P. aeruginosa* can convert pyruvate to lactate, acetate, and succinate, a process which contributes to long-term anaerobic survival (9). It was suggested that ethanol can also be produced as a pyruvate fermentation by-product; however, ethanol production was not detected (9). These observations and the absence of pyruvate in the growth media of our experiments argue against significant ethanol production in the biofilms.

If ethanol production in *P. aeruginosa* biofilms is not responsible for the gene expression changes, then what is responsible? Expression of the ethanol oxidation genes is regulated by three sensor kinases, three response regulators, and additional regulators that have been postulated (22). This complex regulatory scheme implies that several physiological signals, the natures of which are unknown, are integrated. Intriguingly, inactivation of several enzymes involved in central metabolism inhibits the induction of ethanol oxidation genes, suggesting that some internal metabolites are involved in the induction (12, 22). Our results indicate that an *ndvB*-dependent signaling function that can induce expression of the ethanol oxidation genes is found in the *P. aeruginosa* periplasm (Fig. 5), though NdvB-derived glucans themselves do not appear to be the signal. It is possible that a signaling function associates with the glucans but was lost during the glucan purification procedure.

An interesting finding of this study was the antibiotic sensitivity found in several mutants of ethanol oxidation genes. The sensitivity was observed only when the bacteria grew as biofilms. Furthermore, double mutants (Δ*ndvB* plus deletion of *exaA*, *pqqC*, or *erbR*) were more sensitive than the corresponding single mutants. Together, these results suggest that *ndvB* promotes antibiotic resistance in two ways: drug sequestration by cyclic glucans (21) and activation of ethanol oxidation genes. The mechanism by which the ethanol oxidation genes promote antibiotic resistance is unclear but may be related to the redox activity of the ethanol dehydrogenase enzyme. Several studies have demonstrated that PQQ, the cofactor of the dehydrogenase, can protect bacteria from oxidative damage (24, 31). Since bactericidal antibiotics are thought to kill bacteria in part by increasing oxidative stress (17), perhaps PQQ and the ethanol dehydrogenase alleviate antibiotic-induced oxidative stress. Future investigations based on these findings may reveal the nature of this protective mechanism and illuminate the role of cyclic glucans and ethanol oxidation genes in *P. aeruginosa* biofilms.

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