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FosB, a Cysteine-Dependent Fosfomycin Resistance Protein under the Control of σ^W, an Extracytoplasmic-Function σ Factor in *Bacillus subtilis*

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We demonstrate that the *Bacillus subtilis fosB(yndN)* gene encodes a fosfomycin resistance protein. Expression of *fosB* requires σ^W, and both *fosB* and *sigW* mutants are fosfomycin sensitive. FosB is a metallothiol transferase related to the FosA class of Mn^{2+}-dependent glutathione transferases but with a preference for Mg^{2+} and L-cysteine as cofactors.

Sequencing of the *Bacillus subtilis* genome revealed the presence of seven new σ factors, all members of the extracytoplasmic function subfamily (12, 13). We have begun to investigate their functions by mutation of each gene and the identification of target operons (8–11). In this work, we demonstrate that *yndN* encodes a fosfomycin resistance (Fos^r) protein that depends on σ^W for expression. We have renamed *yndN* as *fosB*, based on its similarity to the *fosB* gene identified on a *Staphylococcus epidermidis* plasmid (Fig. 1B).

Transcription of *fosB* requires σ^W. Previously, 15 σ^W-dependent operons were identified by searching the genome for sequences matching the σ^W autoregulatory site, P_w: TGAAAC N_{16} CGTA (10). Additional candidate promoters, including one for *fosB* (Fig. 1A), were identified with 17-bp spacer regions (10).

To confirm the role of this predicted σ^W-dependent promoter, we generated a P_{fosB}-*cat-lacZ* operon fusion inserted ectopically in the SPβ prophage (HB8083; Table 1) and transduced the reporter fusion into wild-type, *sigW*, and *rsiW* mutant backgrounds. Promoter activity as determined in early-stationary-phase cells yielded 18.4 Miller units of β-galactosidase in the wild-type strain (HB0052), and this was reduced to background levels (~1 unit) in the *sigW* mutant (HB0023). In the *rsiW* (anti-σ^W) mutant (HB0012), expression was elevated approximately twofold (30.5 units). This pattern is precisely that expected for a σ^W-dependent promoter.

We used reverse transcriptase primer extension mapping to identify the transcriptional start site for *fosB* as a G residue 10 bases downstream from the −10 region CGTA motif (Fig. 2). There were no other start sites visible in the primer extension experiment, which is consistent with the idea that σ^W is largely, if not exclusively, responsible for *fosB* transcription. The *fosB* gene is apparently monocistronic, as it is flanked on either side by genes transcribed from the complementary strand of the genome (Fig. 1A).

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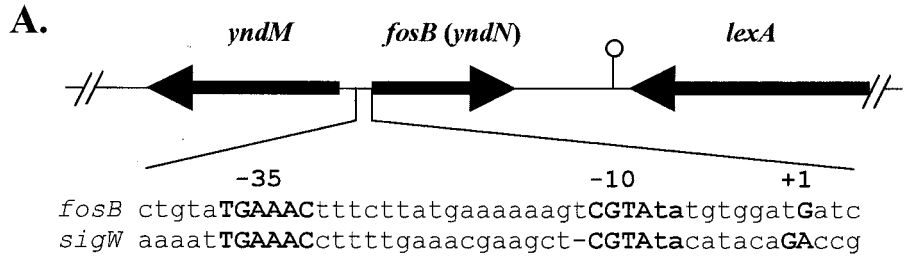
***fosB* and *sigW* mutants are sensitive to fosfomycin.** Both *fosB* (HB0008) and *sigW* (HB0020) mutants are fosfomycin sensitive: an MIC of 50 μg/ml for the mutants compared to 800 μg/ml for the wild type in liquid culture. Similarly, the *sigW* and *fosB* mutants have a much greater zone of growth inhibition in disk diffusion assays (~25-mm zone for wild type versus >50 mm for the mutants). The *fosB* and *sigW* mutant strains did not display altered sensitivity to several other antibiotics, including vancomycin, cephalosporin C, penicillin G, D-cycloserine, tunicamycin, nisin, and bacitracin. Induction of *fosB* from a xylose-inducible promoter completely restores Fos^r to either the *sigW* mutant (HB0081) or, as expected, to the *fosB* mutant (HB0082). Thus, *fosB* is the only σ^W-dependent gene required for wild-type levels of Fos^r.

Expression of FosB confers fosfomycin resistance to *Escherichia coli*. For mechanistic studies, we overproduced FosB in *E. coli*. Transformation of *E. coli* BL21/DE3(pLysS) with pMC50 leads to high fosfomycin resistance, even under noninducing conditions (MIC > 20 mg/ml, as judged using commercial antibiotic disks [Becton Dickinson, Cockeysville, Md.]). This is comparable to the Fos^r imparted by a similar FosA overexpression plasmid (MIC > 30 mg/ml). This suggests that an appropriate thiol cofactor for FosB is present in *E. coli*. To compare the relative efficacy of FosA and FosB in protecting *E. coli* against fosfomycin, the MIC determinations were repeated using plates containing 20 mM glucose-6-phosphate (an inducer of fosfomycin uptake). Under these conditions, FosA still supported high-level fosfomycin resistance (MIC, >30 mg/ml), while resistance of the strain expressing FosB was dramatically reduced (MIC, ~1.25 mg/ml). This difference may relate to the lower catalytic efficiency of FosB compared to FosA (see below).

FosB: an Mg^{2+}-dependent cysteine thiol transferase. FosB was purified using modifications of the FosA procedure (3). Gel filtration chromatography indicated that FosB is a dimer in either the presence or absence of divalent metal ions. The metal ion selectivity of FosB was examined and found to be $Ni^{2+} \sim Mg^{2+} > Mn^{2+} > Fe^{2+} > Cu^{2+} > Ca^{2+} \sim Co^{2+} > Zn^{2+}$ when screened with a fixed (0.5 mM)



B.

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Bsu      MEIKGINHLLFSVSHLDTSIDFYQKVFGAKLLVKGRTTAYFDMNGIWLALNEEPD---IPRNDIKLSYTH 67
Sep       MIKGINHITYSVSNIAKSIEFYRDILGADILVEGETSAYFNLGGIWLALNEEKN---IPRSEIKYSYTH 66
Tn2921    MLQSLNHLTLAVSDLQKSVTFWHELLGLTLHARWNTGAYLTCGDLWVCLSYDEARQYVPPQES--DYTH 67

IAFTIEDHEFEEMSAKLKRLHVNILPGRERDERDRKSIYFTDPDGHKFEFHTGTLQDRLRYYKQEKTHMHFYDETAF 144
IAFTISDNDFEDWYIWLKENEVNILEGRDRDIRDKKSIYFTDLDGHKLELHTGSLEDRLSYYKEAKPHMNFYI    139
YAFTVAEEDFEPLSQRLEQAGVTIW---KQNKSEGASFYFLDPDGHKLELHVGSLAARL                  123
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FIG. 1. The *fosB(yndN)* gene encodes a fosfomycin resistance protein. (A) The *fosB(yndN)* region of the chromosome is illustrated. The *fosB* gene is transcribed from a σ^W-dependent promoter similar in sequence to the *sig*^{W} autoregulatory promoter, P_w (9). (B) Multiple sequence alignment of the 144-amino-acid *B. subtilis* FosB protein (Bsu) with FosB from *S. epidermidis* (63% identity [21]) and with FosA from Tn*2921* (33% identity [14]). Residues identical in all three protein sequences are shown in bold, and those residues identical between the more closely related FosB homologs are underlined in the *B. subtilis* sequence.

concentration of metal. Activation was almost 10-fold greater with Ni^{2+} and Mg^{2+} (Fig. 3) than with Mn^{2+}. Although Ni^{2+} activates the enzyme at lower concentrations, the activation constant for Mg^{2+} (200 μM) is well below the prevailing Mg^{2+} concentration of about 1 mM. Therefore, we suggest that Mg^{2+} is the physiologically relevant metal. In contrast to FosA (2), FosB does not require a monovalent cation for optimal activity.

FosA functions as a glutathione transferase (3). Since *B. subtilis*, like many gram-positive bacteria, lacks detectable levels of glutathione (5, 15), it seems likely that FosB must use a different thiol. To identify the FosB thiol cofactor, the rate of appearance of conjugates of fosfomycin with various thiols was determined as described previously (3, 4). L-Cysteine and coenzyme A sulfhydryl (CoASH) are two abundant thiols in gram-positive bacteria (5, 15). Of the two, only L-cysteine supports a modest enzyme activity (Table 2). The product of the FosB-catalyzed addition of L-cysteine to fosfomycin, examined by heteronuclear multiple-bond correlation nuclear magnetic resonance spectroscopy, is (1*R*,2*S*)-1-(*S*-L-cysteinyl)-2-hydroxypropylphosphonate (data not shown), identical to the product produced by FosA with L-cysteine (4). Extended incubations of FosB, fosfomycin, and various divalent metal ions indicated that no degradation of the antibiotic occurred in the absence of a thiol substrate.

Although with glutathione the catalytic efficiency of FosB is significantly less than that of FosA (k_{cat}/K_m = [1.7 ± 0.3] × 10^5 M^{-1} s^{-1}), they are about equally active with L-cysteine (k_{cat}/K_m = 410 ± 40 M^{-1} s^{-1}). These rate constants are still at least 10^6 to 10^8 greater than those reported for the spontaneous reactions (2). The optimal catalytic efficiency of FosB (with L-cysteine) is far lower than FosA efficiency (with glutathione), which may reflect an intrinsic difference in catalytic efficiency or could indicate that a physiologically relevant cofactor for FosB has not yet been identified. To verify that L-cysteine is the thiol donor in vivo, it will be necessary to characterize the product of fosfomycin inactivation from intact cells.

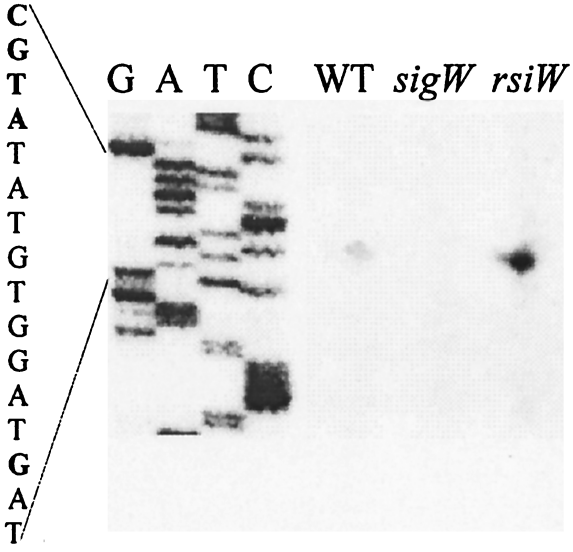

FIG. 2. Expression of *fosB* depends on σ^W. Primer extension analysis of the *fosB* transcription start site is shown. RNA was isolated from logarithmically growing cells in Luria-Bertani medium of strains CU1065 (wild type), HB0020 (*sigW* mutant), and HB0010 (*rsiW* mutant). Equal amounts (100 μg) of total RNA were annealed with radiolabeled oligonucleotide no. 370 prior to analysis by reverse transcriptase primer extension. The transcription start site is the G residue shown in bold. G,A,T, and C are the sequencing ladder obtained with the same primer.

TABLE 1. *B. subtilis* strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Genotype or characteristics[a]	Reference, source, or derivation[b]
Strains		
CU1065	W168 *trpC*2 attSPβ	Lab stock
JH642	W168 *trpC*2 *pheA1*	Lab stock
ZB307A	W168 SPβc2Δ2::Tn*917*::pSK10Δ6; MLS^r	22
HB4247	JH642 *sigW*::*kan*	pKF90→JH642
HB0020	CU1065 *sigW*::*kan*	HB4247→CU1065
HB0010	CU1065 *rsiW*::*kan*	pXH51→CU1065
HB0008	CU1065 *fosB*::pAG4041 (Cm^r)	pAG4041→CU1065
HB8083	ZB307A SPβ P_{fosB}-*cat-lacZ* (MLS^r Neo^r)	pAG3839→ZB307A
HB0052	CU1065 SPβP_{fosB}-*cat-lacZ* (MLS^r Neo^r)	Transduction
HB0023	HB0020 SPβ P_{fosB}-*cat-lacZ* (MLS^r Kan^r)	Transduction
HB0012	HB0010 SPβ P_{fosB}-*cat-lacZ* (MLS^r Kan^r)	Transduction
HB0050	CU1065 SPβ P_w-*cat-lacZ* (MLS^r Neo^r)	9
HB0080	CU1065::pMC82 (P_{xylA}-fosB)	This work
HB0081	HB0020::pMC82; *sigW*::*kan* (P_{xylA}-fosB)	This work
HB0082	HB0008::pMC82; *fosB*::*cat*(P_{xylA}-fosB)	This work
Plasmids		
pET17b	T7 RNAP driven overexpression plasmid	Novagen
pXT	Derivative of pDG1731; allows fusion of genes to xylose-inducible *xylA* promoter; integrates at *thrC* locus	T. Msadek
pJPM122	Vector for integration of reporter fusions into SPβ (Ap^r Neo^r)	18
pJM114	Kanamycin resistance cassette vector	16
pGEM-cat-3Zf(+)	Cloning vector	20
pDG783	Kanamycin resistance cassette vector	6
pKF84	Contains *sigW*	Lab stock
pKF90	Contains *sigW*::*kan*	Construction analogous to *sigW*::MLS (reference 9)
pXH50	pGEM-cat-3Zf(+) carrying *rsiW*	Lab stock
pXH51	*rsiW*::*kan* in pGEM-cat-3Zf(+)	Lab stock
pAG4041	pGEM-*cat*-3Zf(+) carrying internal fragment (330 bp) of *fosB* (PCR primers 275, 276)	This work
pAG3839	pJPM122 carrying P_{fosB} (PCR from 273, 274)	This work
pMC82	pXT carrying *fosB* (PCR from 470, 309)	This work
pMC50	pET17b with *fosB* (PCR from 308, 309)	This work
Oligonucleotides[c]		
275	CGGAATTCAGTTTCGCATTTGGATACA	*fosB* (internal; F)
276	CGTCTAGAAGCCTGTCTTGAAGGGTT	*fosB* (internal; R)
273	GCGAAGCTTCCGTTTTTGTTTACACTGGTA	*fosB* promoter F
274	GCGGATCCTAGCAAGTGATTGATTCCTTTTA	*fosB* promoter R
470	CGCGGATCCATTCATAATGGTCATGTT	*fosB* F
309	CCGGAATTCTGGTTGTGCTATCAAA	*fosB* R
370	CCCTTTACCAAAAGCTTTGCACC	*fosB* (for primer extension; R)
308	CTAGTCTAGACAGTCCGTTTTTGTT	*fosB* (for overproduction; F)

[a] MLS, macrolides-lincosamides-streptogramin B; Neo, neomycin; Kan, Kanamycin, Ap, ampicillin; Cm, chloramphenicol.
[b] Arrows indicate transformation with either plasmid or chromosomal DNA as indicated.
[c] Introduced restriction sites used for cloning are underlined. F, forward; R, reverse (relative to *fosB*).

Summary. Fosfomycin is a clinically important, broad-spectrum antibiotic that irreversibly inactivates MurA, which catalyzes the first committed step in peptidoglycan biosynthesis (17). Resistance arises predominantly via mutations in the chromosomally encoded transport pathways (7) or by resistance genes found on transmissible plasmids. At least two classes of plasmid-borne Fos^r determinants have been described (reviewed in reference 19). The best characterized,

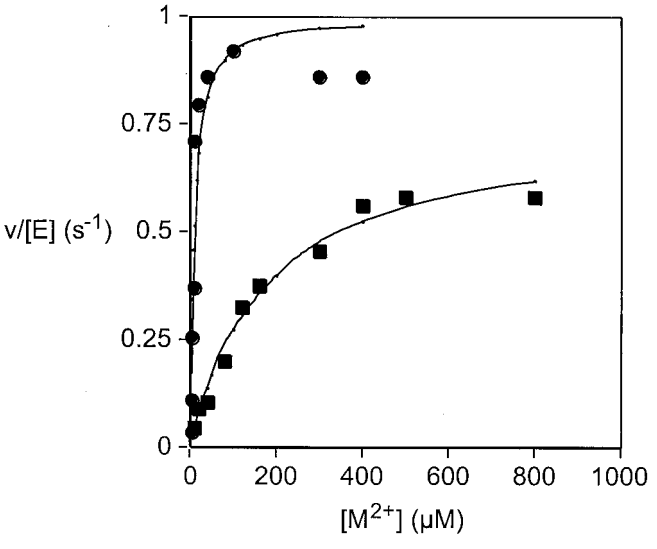

FIG. 3. Activation of FosB with Ni^{2+} and Mg^{2+} using L-cysteine as the thiol substrate. The experimental data for Ni^{2+} (●) and Mg^{2+} (■) were fit to the quadratic form of a simple binding isotherm to obtain the activation constant for the metal (K_{act}^{M}) and the maximum rate constant (k_{max}^{M}) for activation under the conditions of the assay. The solid lines are fits of the experimental data for the two metals, with $K_{act}^{Ni} = 9 \pm 3$ μM, $k_{max}^{Ni} = 1.0 \pm 0.1$ s^{-1}, $K_{act}^{Mg} = 180 \pm 30$ μM, and $k_{max}^{Mg} = 0.75 \pm 0.04$ s^{-1}. For comparison, the results for Mn^{2+} under the same conditions were $K_{act}^{Mn} = 3.5 \pm 0.9$ μM and $k_{max}^{Mn} = 0.11 \pm 0.01$ s^{-1}.

TABLE 2. Steady-state kinetic parameters of the thiol substrate and metal-ion selectivity of FosB

Thiol substrate	Metal ion	k_{cat} (s^{-1})	k_{cat}/K_m^{thiol} (M^{-1} s^{-1})	K_m^{thiol} (mM)
L-Cys	Mg^{2+}	6.3 ± 0.3	180 ± 20	35 ± 3
GSH	Mg^{2+}	0.027 ± 0.002	1.8 ± 0.2	15 ± 2
CoASH	Mg^{2+}	—[a]	0.40 ± 0.03	>50
L-Cys	Ni^{2+}	7.8 ± 0.5	190 ± 30	41 ± 3
GSH	Ni^{2+}	0.066 ± 0.005	9.1 ± 1.7	7 ± 1
L-Cys	Mn^{2+}	—[a]	6.9 ± 0.2	>200
GSH	Mn^{2+}	—[a]	0.093 ± 0.001	>50
CoASH	Mn^{2+}	—[a]	0.0009 ± 0.0001	>100

[a] A linear dependence on the thiol substrate concentration was observed, precluding a determination of k_{cat}. Therefore, a conservative lower limit for K_m^{thiol} was estimated from the substrate concentration range used.

fosA, encodes a Mn^{2+}-dependent glutathione transferase (1–3). A related resistance gene, *fosB*, is from an *S. epidermidis* plasmid. FosB is 38% identical to FosA, suggesting a similar mechanism of action (21), as confirmed in this study. Indeed, the plasmid-borne *fosA* resistance determinants may have arisen from chromosomal genes, like *fosB*, that serve a defensive role within soil microorganisms such as *B. subtilis*.

There are several significant mechanistic differences between FosA and FosB. FosB, unlike FosA, does not function efficiently with glutathione and instead appears to use L-cysteine as the physiological thiol donor. Analyses of *S. epidermidis* FosB have also indicated that glutathione is not involved in detoxification (R. Asano and J. Davies, personal communication). A second mechanistic difference is that FosB uses Mg^{2+} rather than Mn^{2+} as metal cofactor. The final notable difference between FosA and FosB is catalytic efficiency. FosA exhibits a very high catalytic efficiency (k_{cat}/K_m), perhaps in response to selection pressures imposed by the clinical use of fosfomycin.

The assignment of *fosB* to the σ^W regulon further validates the "consensus search" approach for defining alternative σ factor regulons (9–11), and it supports the emerging picture of σ^W as a regulator of a broad, "antibiosis" regulon involved in both the production of, and defense against, antimicrobial compounds.

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