FemABX Family Members Are Novel Nonribosomal Peptidyltransferases and Important Pathogen-specific Drug Targets*

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Pathogen-specific antibiotics kill the offending species without invoking the patient’s flora to help develop a resistance mechanism. The current scarcity of pathogen-specific antibiotics reflects the rarity of essential genes that are also not widely represented in and conserved among species. The FemX enzyme that initiates the synthesis of the interchain peptide of the peptidoglycan in a subset of bacterial species was purified from Lactobacillus viridescens. Subsequently, the encoding femX gene was cloned and sequenced using reverse genetics. The femX gene is a member of the femAB family, a large family of genes previously implicated in interchain peptide synthesis but with unknown specific functions. Mutagenesis of the femX gene identified the members of the extended FemABX family as novel nonribosomal peptidyltransferases. Determinants of FemX complex substrate recognition and a strong stimulator of FemX activity were also identified. The FemABX family members are ideal candidates for pathogen-specific antibiotic development.

Antibiotic resistance in pathogenic bacteria is reaching alarming levels as exemplified by the emergence of vancomycin-resistant Enterococci and Staphylococci (1, 2). This phenomenon is accelerated by the extensive use of broad-spectrum antibiotics, which do not discriminate between targeted pathogens and nontargeted natural flora (2). This situation places critical importance on the identification of new antibiotic targets and the development of new antibiotics. Of particular interest are targets that allow for the development of pathogen-specific antibiotics as pathogen-specific drugs are expected to have both extended clinical lifetimes and increased benefit to risk ratios (3). The enzymes that synthesize the interchain peptide of peptidoglycan are intriguing pathogen-specific antibiotic targets because the interchain peptide: (a) is an essential component of the cell wall, (b) requires several enzymatic steps allowing interchain peptide terminators to be developed as antibiotics, (c) is synthesized in only a subset of Gram-positive species where it varies in both length and sequence, and (d) is synthesized in very few Gram-negative species, including the pathogenic spirochetes but excluding the nonpathogenic enterobacteriaceae of the flora (4).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY098262.
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The peptidoglycan fraction of the bacterial cell wall contains linear chains of alternating N-acetylglucosamine and N-acetylmuramic acid (see “Disaccharide” in Fig. 1). The N-acetylmuramic acid moieties are linked via a lactyl group to the pentapeptide (1-ala)-(d-glu)-x-(1-ala)-(1-ala); where x is the diaminoc acid meso-diaminopimelic acid (Dap), Lys, or ornithine (Orn), depending on species. The pentapeptides in neighboring disaccharide chains are cross-linked to each other either directly or via the interchain peptide, depending on the species. In contrast to other nonribosomal peptide synthesis, interchain peptide synthesis proceeds via aminoaacetyl-tRNA intermediates (5). It is not known whether the interchain peptide is synthesized directly from aminoaacetyl-tRNAs or whether the aminoaacetyl residues are first captured as acylenzyme intermediates. The final cross-linking step in cell wall synthesis occurs via a transpeptidation reaction that displaces the terminal d-Ala of the pentapeptide (Fig. 1). Transpeptidation is the essential cellular process inhibited by both β-lactam antibiotics and vancomycin (6). In addition, the interchain peptide acts as an anchor for the surface proteins that play important roles in adhesion and pathogenicity by interacting with host matrix proteins (7).

Wild-type Staphylococcus aureus cells synthesize a Gly3 interchain peptide. S. aureus femA mutants and femAB double mutants synthesize the truncated interchain peptides Gly4 and Gly5, respectively (8–10). A third gene named femX is postulated to be essential for initiation of the interchain peptide. The existence of additional femAB family members beyond bona fide femA and femB in bacterial genomes and femAB genes in species that synthesize only a single residue interchain peptide suggests that FemX might be encoded by a femAB homolog (11, 12). One of the extra femAB family members from S. aureus named fnhb is essential for cell viability, and inhibition of its synthesis results in a drastic accumulation of uncross-linked UDP-MurNAc pentapeptide precursors of peptidoglycan. Those data are consistent with fnhb assignment as femX (13). However, to date no enzymatic activity has been demonstrated for FnhB or any other FemAB family member.

FemX reactions fall into two classes depending on the enzyme solubility and whether the formation of UDP-MurNAc hexapeptide follows conjugation of UDP-MurNAc pentapeptide to a carrier lipid. In S. aureus and most other characterized species, FemX activity purifies as a large multienzyme “particle” that acts after conjugation of its UDP-MurNAc pentapeptide substrate to the carrier lipid (14). This statement follows from the isolation of only UDP-MurNAc pentapeptide from S. aureus cytoplasm, the substrate of FemX reaction (15). By

1 The abbreviations used are: Dap, meso-diaminopimelic acid; Orn, ornithine; IPTG, isopropyl-1-thio-β-D-galactopyranoside; PCR, polymerase chain reaction; contigs, groups of overlapping clones.
contrasts, FemX activity in the naturally vancomycin-resistant species Lactobacillus viridescens (ATCC 12706) is a soluble enzyme that acts before conjugation to the carrier lipid (16). This statement follows from our isolation of UDP-MurNAc hexapeptide from the cytoplasm of L. viridescens, the product of the FemX reaction (see below). Here we report the purification of L. viridescens FemX, identification of the femX gene, recombinant expression of active FemX, a partial characterization of FemX complex substrate specificity, and a strong stimulator of FemX activity from Escherichia coli. 

FemABX Family Members

**FemX Assays—**FemX activities were assayed by monitoring the incorporation of [14C]Ala into purified UDP-MurNAc pentapeptides. FemX assays were carried out according to Flapp and Strominger (16) with minor modifications. Fifty-microliter assay mixtures contained 30 µM UDP-MurNAc pentapeptide, 500 µM ATP, 50 µg of E. coli tRNA (Sigma) (18 µM [14C]Ala (Amersham Pharmacia Biotech)), 50 units of E. coli aminocyl-tRNA synthetase (Sigma), 2 µl of appropriately diluted FemX in 50 mM Tris buffer, pH 7.6, 20 mM MgCl₂, and 50 mM NaCl. FemX reactions were incubated at room temperature for 30 min and stopped by the addition of 1 µl of 2.0 mM NaOH. 3 µl of each reaction mixture was spotted onto precoated cellulose TLC plates (Selecto Sci- ence), fractionated using ethanold 1 M ammonium acetate (75.3 µl v/v) as the solvent. Spots were visualized by autoradiography, excised, and quantitated by counting in liquid scintillation fluid. One unit of enzyme activity is defined as the amount of the protein that catalyzes the incorporation of 1 nmol of [14C]Ala into UDP-MurNAc pentapeptide per min under standard assay conditions. FemX assays were carried out as described above but used UDP-MurNAc hexapeptides isolated from L. viridescens as the acceptor substrate (see above).

**Isolation of UDP-MurNAc Pentapeptides—**UDP-MurNAc pentapeptides from bacterial cultures were isolated and purified as described previously (15) with DEAE-Sepharose and Sephadex G-10/Bio-Gel P-2 chromatography being substituted in our purification scheme. UDP-MurNAc hexapeptides, UDP-MurNAc heptapeptides, and UDP-MurNAc octapeptides from L. viridescens cultures were isolated using the same procedures. S. aureus cultures were grown to mid-log phase (A₆₀₀ = 1.0), vancomycin was added to a concentration of 5 µg/ml, and the flask was incubated for an additional 3 h. L. viridescens cultures were grown in MRS medium (Difco) at 30 °C to mid-log phase (A₆₀₀ = 1.0), vancomycin was added to a concentration of 5 µg/ml, and the flask was incubated for an additional 3 h. Borellia burgdorferi cultures were grown in BSK-H medium (Sigma) at 30 °C in tightly capped bottles under mild agitation (40 rpm). Vancomycin was added to a final concentration of 0.4 µg/ml after 84 h, and incubation was continued for 48 h. An additional aliquot of vancomycin (0.4 µg/ml) was added and incubated for 24 h. E. coli cultures containing the femX gene (pQE-12) were grown in LB medium. FemX expression was induced by the addition of 25 µM IPTG when the A₆₀₀ reached 0.6 followed by incubation for an additional 3 h.

**Electrospray Ionization Mass Analysis—**Mass analysis of UDP-MurNAc peptides and larger species isolated from bacteria and FemX reaction products were carried out using a Finnigan LCQ mass spectrometer in a negative ion mode.

**Identification of the femX Gene and Construction of Recombinant Expression Systems—**Degenerate deoxyoligonucleotide primers were synthesized based on the NH₂-terminal peptide and the two internal peptides. PCR using these primers amplified regions of L. viridescens genomic DNA to generate products of 450 and 950 base pairs, respectively. The larger of these fragments was cloned and sequenced. This fragment of FemX was enzymatically active when expressed with COOH-terminal His₆ tag. Tosubjected to automated Edman degradation to obtain NH₂-terminal (N) and internal peptide (IP) sequence information: N = PVILNLND-PQAVERYEEFMQRQSPY, IP1 = TLTDLQFPK2, and IP2 = EYIIEID-KVLDPEVYAE1. None of these peptides had significant matches in available sequence data bases or with any of the known FemAB family members.

To identify the femX gene and construct recombinant expression systems, degenerate deoxyoligonucleotide primers were designed to express FemX bearing an NH₂-terminal His₆ affinity tag (BamHI fragment for expression in pQE8, Qiagen), a COOH-terminal His₆ affinity tag (EcoRI/BamHI fragment for expression in pQE12, Qiagen), and without an affinity tag (EcoRI/BamHI fragment in pQEI2). Primers for expression of COOH-terminal His₆-tagged FemX in pQEI2 were LFQ12N = CCCCGCCTGATTCAATAGGGAGAATAACTATGCCTGATTTAAT and LFCH6 = CCCGCTGGATCACCTATTTAACTATTAATTC. Primers for expression of wild type FemX in pQE12 were: LFQ12N = CCCGCTGAA-TTCTATTAAAGGAGAAATAACTATGCCTGATTTAAT and LFCH6 = CCCGCTGGATCACCTATTTAACTATTAATT. Primers for expression of NH₂-terminal His₆-tagged FemX in pQE8, LFQ12N = CCCGCTGGATCACCTATTTAACTATTAATTC. Primers are written from their 5′-3′ ends.

**Recombiant Expression of FemX and Its Homolog—**FmB (GenBank™ accession number AF106830) was expressed bearing an NH₂-terminal His₆ affinity tag (BglII fragment for expression in pQE8), a
COOH-terminal His$_6$ affinity tag (EcoRI/BglII fragment for expression in pQE12), and without any modifications (EcoRI/BglII fragment for expression in pQE12). Three FemAB homologs from \textit{Streptococcus pyogenes}, 254–1, 297–2, and 297–3, were expressed with NH$_2$-terminal His$_6$ affinity tags (BamHI fragments for expression in pQE8; numbers refer to current contigs as the sequencing of the \textit{S. pyogenes} genome is in progress).

\textbf{FIG. 2. Sequence alignment of the FemABX family.} Sequence comparisons employed the following sequences: 1) \textit{L. viridescens} FemX, 2) \textit{S. aureus} FemB (GenBank\textsuperscript{TM} accession number AF106850), 3) \textit{S. aureus} FemA (GenBank\textsuperscript{TM} accession number X17688), 4) \textit{Staphylococcus capitis} Epr (GenBank\textsuperscript{TM} accession number AB000222), 5) \textit{Staphylococcus simulans} Lif (GenBank\textsuperscript{TM} accession number U66883), 6) \textit{B. burgdorferi} FemAB family member (GenBank\textsuperscript{TM} accession number AE001160) 7 and 8) two \textit{S. pyogenes} FemAB family members (254–1 and 297–2, numbers refer to current contigs as the sequencing of the \textit{S. pyogenes} genome is in progress).
in progress). All clones were transformed into E. coli strain DH5α containing pREP4 (Qiagen). Recombinant proteins bearing His₆ affinity tags were purified using immobilized metal affinity chromatography using nickel-nitrilotriacetic acid agarose according to the manufacturer’s instructions (Qiagen). Purified enzymes and lysates were assayed for FemX activity as described above.

Site-directed Mutagenesis—Site-directed mutagenesis was carried out using the Quick Change site-directed mutagenesis kit (Stratagene). Deoxynucleotide synthesis and other procedures were performed according to the manufacturer’s instructions. COOH-terminal His₆-tagged mutants were purified using immobilized metal affinity chromatography that used nickel-nitrilotriacetic acid agarose as described above.

Identification of a FemX Stimulator from E. coli—Cultures of E. coli strain DH5α were grown to an A₆₀₀ of 1.5, and the cells were lysed by sonication. Cell lysate in 50 mM Tris buffer, pH 7.6, was adsorbed onto DEAE-Sephacel and washed with 0.15M NaCl. The stimulating activity was eluted with 0.25 M NaCl. The stimulating activity was further fractionated on phenyl-Sepharose in the above buffer containing 1.7 M ammonium sulfate. Stimulating activity was eluted with 0.6–0.4 M ammonium sulfate and concentrated. Finally, the stimulating activity was fractionated on Sephacryl S-200 where it eluted in the void volume.

RESULTS AND DISCUSSION

FemX Is a Homolog of FemA and FemB—We purified FemX from L. viridescens and cloned its encoding femX gene using reverse genetics. Data base searches identified femX (GenBank™ accession number AY008262) as a member of the femABX family (Fig. 2). The femABX family shows no homology to the leucine/phenylalanine transferase family; the only other known example of a bacterial enzyme that synthesizes a peptide bond using an aminoacyl-tRNA substrate (17). Sequence data upstream and downstream of the femX coding region (3–500 base pairs each way) revealed that femX is not a part of an operon. Recombinant expression of FemX yielded a soluble enzyme that initiated synthesis of an interchain peptide in vitro (Fig. 3A). Recombinant FemX had a molecular mass of 39,360, which is in progress.

Fig. 3. A, ESI mass spectrum of a FemX reaction carried out with a mixture of alanine-, serine-, and glycine-tRNAs. Relevant masses are: UDP-MurNAc pentapeptide = 1149; UDP-MurNAc hexapeptide (glycine addition) = 1206; UDP-MurNAc hexapeptide (alanine addition) = 1220; UDP-MurNAc hexapeptide (serine addition) = 1236. B, recombinant FemX activity. FemX reactions carried out with UDP-MurNAc pentapeptides containing lysine (Lys), Dap, or Orn in the X position (see text). Units are counts. 2000 counts corresponds to 0.17 nmol of product. The first bar corresponds to assays lacking aminoacyl-tRNA synthetase additions (background).

TABLE I
Specific activity of FemX mutant enzymes

| FemX Activity | FemX Activity |
|---------------|---------------|
| %             | %             |
| Wild type     | 100           | P110L | ND |
| Q29E          | ND            | P110H | ND |
| Q29T          | ND            | P110F | ND |
| Y73F          | 40            | F196L | 26 |
| Y73L          | 40            | F196Y | 87 |
| Q144E         | 79            | G292V | ND |
| Q144T         | 79            | F305Y | 98 |
| Y216F         | 41            | F305L | 16 |
| Y216L         | ND            | G319L | 5  |
| K306R         | 25            | G319S | 13 |
| K306C         | 38            |       |     |
| K306A         | 8             |       |     |
| K306N         | 9             |       |     |

Identification of a FemX Stimulator from E. coli— Cultures of E. coli strain DH5α were grown to an A₆₀₀ of 1.5, and the cells were lysed by sonication. Cell lysate in 50 mM Tris buffer, pH 7.6, was adsorbed onto DEAE-Sephacel and washed with 0.15 M NaCl. The stimulating activity was further fractionated on phenyl-Sepharose in the above buffer containing 1.7 M ammonium sulfate. Stimulating activity was eluted with 0.6–0.4 M ammonium sulfate and concentrated. Finally, the stimulating activity was fractionated on Sephacryl S-200 where it eluted in the void volume.

Recombinant FemX activity. FemX reactions carried out with UDP-MurNAc pentapeptides containing lysine (K) or Orn (O) in the X position. Specific activities of FemX for UDP-MurNAc pentapeptide containing lysine and Orn were 55 and 28 units, respectively. Specific activities in the presence of stimulator were 220 and 110 units, respectively.

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Fig. 4. Stimulation of FemX reactions by fractionated E. coli lysate. Reactions were carried out ± 0.2 µg of fractionated E. coli lysate. X/K = FemX reactions (X) ± lysine (L) employing UDP-MurNAc pentapeptides containing lysine (K) or Orn (O) in the X position. Specific activities of FemX for UDP-MurNAc pentapeptide containing lysine and Orn were 55 and 28 units, respectively. Specific activities in the presence of stimulator were 220 and 110 units, respectively.

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FemX Is a Homolog of FemA and FemB—We purified FemX from L. viridescens and cloned its encoding femX gene using reverse genetics. Data base searches identified femX (GenBank™ accession number AY008262) as a member of the femABX family, now called the femABX family (Fig. 2). The femABX family shows no homology to the leucine/phenylalanine transferase family; the only other known example of a bacterial enzyme that synthesizes a peptide bond using an aminoacyl-tRNA substrate (17). Sequence data upstream and downstream of the femX coding region (3–500 base pairs each way) revealed that femX is not a part of an operon. Recombinant expression of FemX yielded a soluble enzyme that initiated synthesis of an interchain peptide in vitro (Fig. 3A). Recombinant FemX had a molecular mass of 39,360, which is in progress.
agreement with the mass deduced from the *femX* gene. The specific activities of the His<sub>6</sub>-tagged and native enzymes were compared and are equivalent. Purified FemX was soluble to >15 mg/mL, and gel filtration chromatography suggested that the enzyme is a monomer.

**Recombinant FemX Initiates Interchain Peptide Synthesis**—Incubation of FemX with UDP-MurNAc pentapeptide resulted in approximately equal rates of transfer of alanine and serine from their corresponding *E. coli* tRNAs to UDP-MurNAc pentapeptides containing lysine in their X position (see Fig. 1). Glycine was also transferred but at a much lower rate (Fig. 3A). Assays with mixtures of alanine-, serine-, and glycine-tRNAs resulted in mixtures of the expected UDP-MurNAc hexapeptides but no larger species (Fig. 3A). We conclude that FemX adds only the first residue of the interchain peptide. By contrast, recombinant expression of neither *S. aureus* FmhB nor any of the FemABX family members from the *S. pyogenes* showed FemX or FemA activity. Each inactive FemABX enzyme was readily overexpressed and purified from *E. coli* and showed a CD spectrum characteristic of a folded protein. These data suggest that FemX enzymes that purify as large multiprotein complexes require additional factors or lipid conjugation for their activity.

**In Vivo Specificity of FemX**—Partial inhibition of *S. aureus* with vancomycin or β-lactams results in the accumulation of only UDP-MurNAc pentapeptides in the cytoplasmic precursor pools of these cells, the substrates of FemX reaction (15). Those data form the basis for the statement that FemX from *S. aureus* acts after conjugation of UDP-MurNAc pentapeptide to the carrier lipid (15). By contrast, partial inhibition of *L. viridescens* with vancomycin resulted in the accumulation of UDP-MurNAc pentapeptides conjugated in equal proportions to alanine, alanine-serine, and Ala-Ser-Ala as indicated by mass analysis of cytoplasmic precursor pools (mass numbers 1221, 1309, and 1380, respectively). These species are the expected products of FemX and FemX + FemAB reactions (see Fig. 1).

No UDP-MurNAc pentapeptides were detected by mass analyses of *L. viridescens* cytoplasmic precursor pools. In addition, the UDP-MurNAc peptide mixtures isolated from *L. viridescens* were not substrates for the FemX reaction, confirming that no UDP-MurNAc pentapeptides were contained in these mixtures. Importantly, these same precursors were detected in similar proportions in the absence of vancomycin, although at much lower absolute concentrations. Those data reveal that the FemX enzyme from *L. viridescens* acts on UDP-MurNAc pentapeptides before the conjugation of this intermediate to a lipid precursor.

Interestingly, no UDP-MurNAc hexapeptide conjugated to serine was detected in the cytoplasmic precursor pools in *L. viridescens* by mass analysis, either in the presence or absence of vancomycin. The ability of purified recombinant FemX to make this product in vitro (see above) remains unexplained.

**Substrate Specificity of FemX**—*E. coli* cells tolerated the expression of FemX at high levels. This lack of an observed phenotype for *E. coli* cells expressing FemX suggested that UDP-MurNAc pentapeptides containing Dap in the X position were not substrates for FemX because modification of UDP-MurNAc pentapeptide pools in *E. coli* is expected to be highly deleterious (see Fig. 1 for details). These statements follow from experiments demonstrating that: (a) the expression of *murE* from *S. aureus* in *E. coli* cells resulted in the substitution of lysine for Dap at the X position in ~50% of *E. coli* pentapeptides (18); and (b) this relatively minor substitution blocked the transpeptidation reaction and arrested cell growth (18).

This suggestion that Dap in the X position abolished recognition by FemX was confirmed directly. No UDP-MurNAc hexapeptides were detected in the isolated cytoplasmic precursor pools of *E. coli* strains overexpressing FemX. In addition, isolated UDP-MurNAc pentapeptide containing Dap in the X position (isolated from *B. subtilis*, see Fig. 1) was not a substrate for the FemX reaction (Fig. 3B). By contrast, UDP-MurNAc pentapeptide containing Orn in the X position (isolated from *B. burgdorferi*) was a substrate for the FemX reaction (Fig. 3B). Isolated pentapeptides with lysine in the X position were neither substrates nor inhibitors for the FemX reaction in the presence or absence of UDP and/or MurNAc. These data suggest (a) the deletion of a single methylene group from the side chain of lysine does not abolish recognition by FemX, but the addition of an acid group to lysine does and (b) there is a requirement of an intact UDP-MurNAc pentapeptide bond geometry for recognition by FemX.

**FemX Mutagenesis**—Overall, the FemABX family is poorly conserved, and FemX is less than 20% similar to other known FemABX family members (Fig. 2). This low level of sequence conservation is noteworthy in light of the fact that each family member recognizes two large and relatively conserved substrates, an aminoacyl-tRNA and a UDP-MurNAc pentapeptide. Sequence alignments of FemABX family members revealed that the only conserved region greater than a single residue is the sequence Phe-305–Lys-306 (Fig. 2). However, mutagenesis experiments identified Gln-29 as the only conserved and potentially catalytic residue that was essential for FemX activity (Table I). Mutation of the conserved nonpolar residues proline 110 and glycine 292 also resulted in inactive enzyme despite normal yields of recombinant protein (Table I). Those data reveal that there are no absolutely conserved residues that might form acyl-enzyme intermediates in the FemABX family and identifies the FemABX family members as novel nonribosomal peptidyltransferases. The higher FemX activity of mutants F305Y and Y216F, relative to F305L and Y216L, is consistent with the participation of these conserved aromatic residues in an aromatic-aromatic interaction with aminoacyl-tRNA substrates. Despite the low level sequence conservation within the family, the COOH-terminal region of FemX is important for function as truncation by more than 10 residues at this end of the enzyme abolished its activity (data not shown).

**Stimulation of FemX Activity**—Purified FemX was stimulated by *E. coli* cell lysate 4–8-fold (Fig. 4). Fractionation of *E. coli* lysate revealed that the stimulating activity is a protein or a complex of proteins with a molecular mass > 200,000 daltons. Control experiments were performed to further characterize the stimulating activity. The stimulating activity lacked aminoacyl-tRNA synthetase activity. The stimulating activity is not an ATP-dependent protease because FemX was not processed or degraded during assays (assayed by SDS-polyacrylamide gel electrophoresis). The stimulating activity is not the GroESL molecular chaperone because co-overexpression of the GroESL chaperone (17) did not increase the specific activity of FemX from *E. coli* lysates or the resultant purified FemX. The stimulating activity was not tRNA based on the purification scheme, the spectrum of stimulator (A<sub>260 nm</sub> = 0.6), and tRNA addition experiments. Stimulation was not because of general molecular crowding effects because stimulation was not observed for other and more concentrated *E. coli* lysate fractions. Our working model is that the stimulating activity is a UDP-MurNAc pentapeptide binding complex that presents this substrate in an optimal conformation to FemX. This statement follows from: (a) the existence of the stimulator in *E. coli*, a species lacking FemX, and (b) data demonstrating that each of the FemX mutants in Table I were stimulated ~4-fold by fractionated *E. coli* lysate.

This work identifies the members of the FemABX family as
nonribosomal peptidyltransferases. To our knowledge, this is the first demonstration of a specific enzymatic activity for the members of the FemABX family and identifies the members of this family as targets for pathogen-specific antibiotic development.

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