Molecular Cloning, Sequence Analysis, and Characterization of a Penicillin-Resistant DD-Carboxypeptidase of *Myxococcus xanthus*

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Received 11 March 1999/Accepted 20 May 1999

We have cloned a gene, *pdcA*, from the genomic library of *Myxococcus xanthus* with an oligonucleotide probe representing conserved regions of penicillin-resistant DD-carboxypeptidases. The amino- and carboxy-terminal halves of the predicted *pdcA* gene product showed significant sequence similarity to *N*-acetylmuramoyl-L-alanine amidase and penicillin-resistant DD-carboxypeptidase, respectively. The *pdcA* gene was expressed in *Escherichia coli*, and the characteristics of the gene product were similar to those of DD-carboxypeptidase (VanY) of vancomycin-resistant enterococci. No apparent changes in cell growth, sporulation, or germination were observed in *pdcA* deletion mutants.

*Mycococcus xanthus* is a gram-negative bacterium which lives in soil (7, 17, 35). It feeds upon other microorganisms by secreting bacteriolytic enzymes and antibiotics (14, 33, 37). The bacterium responds to nutrient starvation by forming a multicellular aggregate and fruiting body. *M. xanthus* cells coordinate fruiting-body formation by transmitting intercellular signals (18, 22, 34, 38). During the formation of the fruiting body, a morphological change from rod-shaped to spherical cells occurs, and the cells differentiate to form myxospores.

Although low-molecular-weight penicillin-binding proteins (PBPs) of *Escherichia coli* are dispensable for bacterial growth and division (3, 25), the morphological change during stationary phase requires the PBPs DD-transpeptidase and DD-carboxypeptidase (24, 39). In *Bacillus* species, many cell wall hydrolases, such as *N*-acetylmuramoyl-L-alanine amidasates (20, 21) and endopeptidases (15), and DD-carboxypeptidases (36) contribute to sporulation and germination. On the other hand, DD-dipeptidase (VanX) and DD-carboxypeptidase (VanY) of vancomycin-resistant enterococci regulate the synthesis of new resistant peptidoglycan precursors and the elimination of wild-type sensitive peptidoglycan precursors (12, 32). There have been very few investigations dealing with cell morphological enzymes of *M. xanthus*, and the results that have been reported are inconclusive. Recently, we reported that *M. xanthus* produces DD-carboxypeptidases during development (19). In this paper, we report the cloning and sequencing of a penicillin-resistant DD-carboxypeptidase gene, *pdcA*, from *M. xanthus*, comparison of the amino acid sequence of PdcA with those of other penicillin-resistant DD-carboxypeptidases, and the characterization of a *pdcA*-deficient mutant.

**Cloning of *pdcA*-carboxypeptidase gene from *M. xanthus*.** To examine whether *M. xanthus* IFO13542 (ATCC 25232) produces DD-carboxypeptidase, we attempted to clone the *pdcA*-carboxypeptidase gene with appropriate oligonucleotide probes designed from conserved sequences in the DD-carboxypeptidases of PBPs in *E. coli* or penicillin-resistant DD-carboxypeptidases of vancomycin-resistant *Enterococcus*. One positive phage was cloned by hybridization with an oligonucleotide probe (van Y). The sequence of van Y is 5'-CTGGT GCTCG(G)GAC(G)GTGGCCCGG-3' (the nucleotides in parentheses are degenerate), which was designed according to the conserved motifs (PGTSEHQ at amino acid positions 181 to 183) of DD-carboxypeptidase (VanY) of vancomycin-resistant enterococci. No apparent changes in cell growth, sporulation, or germination were observed in *pdcA* deletion mutants.

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the Zn\(^{2+}\)-DD-carboxypeptidases (Zn-DD) of *Streptomyces albus* G (6) (23% identity with positions 1 to 140 of Zn-DD) (Fig. 3). The PdcA product contained four short repeated sequences [DGx(F)VGPxO(W)SDA at positions 50 to 61, 73 to 84, 124 to 135, and 147 to 158], and the direct repeats are probably involved in the recognition of repeated units of peptidoglycan of the cell wall (27). Such imperfect direct repeats have been found in noncatalytic regions of various peptidoglycan hydrolases of bacilli (27).

The amino acid sequence of the carboxy-terminal half of the PdcA protein (positions 179 to 302) was similar to those of the DD-carboxypeptidases (VanY and VanY B) of vancomycin-resistant enterococci (1, 8) (21 and 36% identities with positions 71 to 221 and 96 to 246, respectively). Motifs [SxHxxGxA(S)xD and EP(W)WH] conserved in DD-dipeptidases and DD-carboxypeptidases of vancomycin-resistant enterococci (32) were present in the carboxy-terminal half of PdcA (Fig. 3). The PdcA protein did not reveal significant similarity to *E. coli* DD-carboxypeptidases PBP5 and PBP6 (2), and the PdcA protein contained no hydrophobic transmembrane regions.

**DD-carboxypeptidase activity of PdcA.** To investigate the biological function of PdcA, expression plasmid pPDC-T was constructed by subcloning a 2.2-kb *Nco*I fragment containing the *pdcA* gene into a region downstream of the thioredoxin gene (encoding TrxA) in pET-32a(+) (Novagen, Madison, Wis.) and then transferred to *E. coli* BL21(DE3) (Novagen). Formation of the TrxA-PdcA fusion product (48 kDa) was induced by 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 2 h, and the protein was produced in soluble fractions in *E. coli*. While the cells transformed with pET-32a(+) showed low levels of DD-carboxypeptidase activity, cells transformed with pPDC-T produced a large amount of DD-carboxypeptidase (Table 1). The DD-carboxypeptidase activity was impervious to penicillin at concentrations of 5 to 10 mM. The enzyme activity was also not affected by the addition of 5 mM EDTA or Mg\(^{2+}\). Zn-DD (metalloprotease) of *S. albus* G (5) has been reported to be penicillin resistant. Since the PdcA product was not inhibited by 5 mM EDTA, it was not a metalloprotease. The fusion product did not show DD-dipeptidase activity (data not shown). These results indicate that the characteristics of PdcA are similar to those of penicillin-resistant DD-carboxypeptidases of vancomycin-resistant enterococci (40). We are not aware of any reports on penicillin-resistant DD-carboxypeptidases of gram-negative bacteria.

**Characterization of the pdcA mutant.** A kanamycin resistance gene of pTF1 (10) was inserted into the *Stu*I site of the *pdcA* gene. The insertion mutation was moved into the chromosome of *M. xanthus* by the electroporation method of Plaumann et al. (31). Using Southern hybridization and PCR analyses, we confirmed that the kanamycin resistance gene was inserted into the *pdcA* gene on the chromosome of the mutant. To investigate its biological function in *M. xanthus*, the cell morphology, sporulation, and germination of a *pdcA* deletion mutant were examined. The *pdcA* deletion mutant produced normal-sized fruiting bodies of normal shape and spore morphology on clone fruiting (CF) agar (13) (data not shown). Differences in cell morphology or germination between the wild type and a *pdcA* deletion mutant were not observed when vegetative cells or spores were incubated in Casitone-yeast extract (CYE) medium (4).

In vancomycin-resistant enterococci, the *vanY* gene is a member of the vancomycin resistance *van* gene cluster (23). In *VanA*-type enterococci, VanY is nonessential for resistance gene (encoding TrxA) in pET-32a(+) (Novagen, Madison, Wis.) and then transferred to *E. coli* BL21(DE3) (Novagen). Formation of the TrxA-PdcA fusion product (48 kDa) was induced by 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 2 h, and the protein was produced in soluble fractions in *E. coli*. While the cells transformed with pET-32a(+) showed low levels of DD-carboxypeptidase activity, cells transformed with pPDC-T produced a large amount of DD-carboxypeptidase (Table 1). The DD-carboxypeptidase activity was impervious to penicillin at concentrations of 5 to 10 mM. The enzyme activity was also not affected by the addition of 5 mM EDTA or Mg\(^{2+}\). Zn-DD (metalloprotease) of *S. albus* G (5) has been reported to be penicillin resistant. Since the PdcA product was not inhibited by 5 mM EDTA, it was not a metalloprotease. The fusion product did not show DD-dipeptidase activity (data not shown). These results indicate that the characteristics of PdcA are similar to those of penicillin-resistant DD-carboxypeptidases of vancomycin-resistant enterococci (40). We are not aware of any reports on penicillin-resistant DD-carboxypeptidases of gram-negative bacteria.

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and has been reported to control the abundance of peptidoglycan precursors (1). M. xanthus produces the antibiotic TA, which inhibits the polymerization step in cell wall formation, leading to an accumulation of lipid intermediates (41), and its mode of action is similar to that of vancomycin (30). Although no significant differences in growth were also observed between the wild type and pdcA mutants grown in antibiotic TA production medium, 0.5 CT (0.5% Casitone and 0.2% MgSO$_4$·7H$_2$O) (41) (data not shown), this molecule may have a role similar to that of VanY of vancomycin-resistant enterococci. On the other hand, since β-lactamase of M. xanthus is induced by β-lactams (28), PdcA may also play a role in multiple mechanisms to resist β-lactams it encounters in soil. Future work will provide insight into the roles of PdcA in this bacterium.

Nucleotide sequence accession number. The nucleotide sequence data reported here will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases under accession no. AB023893.

This work was supported in part by a grant-in-aid for scientific research (no. 09760305) from the Ministry of Education, Science and Culture of Japan.

REFERENCES


FIG. 3. Similarity of the deduced amino acid sequence of PdcA to an N-acetylmuramoyl-L-alanine amidase and penicillin-resistant ß-carboxypeptidases: CwlL (29); Zn-DD (6); VanY, E. faecalis V583 ß-carboxypeptidase (8); and VanY, E. faecium BM4147 ß-carboxypeptidase (1). The conserved motifs of the ß-carboxypeptidases are boxed; identical residues are shaded. Dashes indicate spaces introduced to maximize alignment.

B. Carboxy-terminal half of PdcA

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Fusion product induction</th>
<th>Total activity (mU)</th>
<th>Sp act (mU/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET-32a(+)</td>
<td>–</td>
<td>3.0</td>
<td>0.6</td>
</tr>
<tr>
<td>pPDC-T (pET-32a+pdcA)</td>
<td>–</td>
<td>3.0</td>
<td>0.6</td>
</tr>
<tr>
<td>pET-32a(+)</td>
<td>+</td>
<td>5.0</td>
<td>1.2</td>
</tr>
<tr>
<td>pPDC-T (pET-32a+pdcA)</td>
<td>+</td>
<td>55.7</td>
<td>16.6</td>
</tr>
</tbody>
</table>

*The ß-carboxypeptidase activity was measured by incubating enzyme extract with 5 mM diacetyl-L-Lys-D-Ala-D-Ala in a final volume of 50 μl for 30 min at 30°C and by estimating the amount of C-terminal D-alanine liberated enzymatically (9). One unit of enzyme catalyzed the hydrolysis of 1 μmol of the substrate per min.

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TABLE 1. ß-Carboxypeptidase activity of enzyme extracts of E. coli harboring pET-32a or pPDC-T*


