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Role of the C-terminal domain of the lysozyme of *Clostridium acetobutylicum* ATCC 824 in a chimeric pneumococcal–clostridial cell wall lytic enzyme

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An active chimeric cell wall lytic enzyme has been constructed by domain substitution between the major autolysins of *Clostridium acetobutylicum* ATCC 824 and *Streptococcus pneumoniae*. The chimeric enzyme, built up by the fusion of the N-terminal domain of the pneumococcal LYTA amidase and the C-terminal domain of the clostridial LYC lysozyme, exhibited an amidase activity capable of hydrolyzing choline-containing clostridial cell walls with an efficiency 250-times higher than when tested on pneumococcal cell walls. This experimental approach demonstrates the basic role of the C-terminal domain of the LYC lysozyme in substrate recognition and provides additional support to our hypothesis of modular evolution of these lytic enzymes. Moreover, the construction described here confirmed the role of the C-terminal domains of the modular cell wall lytic enzymes on the optimal pH for catalytic activity. To our knowledge, this is the first example of the construction of an active chimeric lytic enzyme by fusing genes that lack nucleotide homology and are derived from different bacterial genera.

Autolysis; *Streptococcus pneumoniae*; Chimeric enzyme; Modular evolution

1. INTRODUCTION

The LYC autolysin from *Clostridium acetobutylicum* ATCC 824 has been characterized as a lysozyme [1], one of the enzyme families most widely distributed in nature [2]. The *lyc* gene encoding this enzyme has recently been cloned, sequenced and expressed in *Escherichia coli* [3,4]. It has been suggested that the LYC lysozyme has evolved through the fusion of two independent domains, the N-terminal domain that should contain the catalytic center and the C-terminal domain that may be responsible for the binding of the protein to the substrate [3]. This hypothesis has been partially confirmed by fusing the N-terminal domain of the clostridial lysozyme and the C-terminal domain (choline-binding domain, ChBD) of the CPL1 lysozyme from the bacteriophage Cp-1 of *Streptococcus pneumoniae* [5]. This chimeric enzyme constructed by domain substitution expanded the capacity of the clostridial lysozyme to degrade choline-containing pneumococcal cell walls. The ChBD enables the chimeric enzyme to recognize pneumococcal cell walls as substrate since the native clostridial lysozyme was unable to hydrolyze these walls [5].

Inasmuch as the construction of this chimeric enzyme demonstrated that the N-terminal domain of the LYC lysozyme contains the catalytic domain [5], the aim of the present study was to determine whether the C-terminal of this choline-independent enzyme was involved in cell wall recognition, as already demonstrated for several lytic enzymes of *S. pneumoniae* and its bacteriophages [6–11]. We report here the fusion of the N-terminal domain of the LYTA amidase, the major autolysin from *S. pneumoniae* [6], with the C-terminal domain of the clostridial lysozyme. This approach allowed us to evaluate the influence of the C-terminal domain of the LYC lysozyme on catalytic activity, as well as to expand the hypothesis of modular evolution of most proteins.

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2. MATERIALS AND METHODS

2.1. *Bacterial strains, preparation of radioactively labelled cell walls of* C. acetobutylicum *NCIB 8052 and* S. pneumoniae *and assay of enzymatic activity*

The *E. coli* strains used were DH1 [12] and MC4100 [4]. Plasmids used were pCA33, which contains the DNA fragment encoding the C-terminal domain of the LYC lysozyme [3], and pAC300 [4], pGL100 [13] and pGL300 [11], which express the LYC lysozyme, the LYTA amidase and the N-terminal domain of the LYTA amidase (N-LYTA), respectively, under the control of the strong *lpp–lac* promoter of plasmid pINIII-A3 [14]. *E. coli* extracts were prepared by sonication of cells cultured in LB medium [12] containing ampicillin (100 μg/ml) and 2% lactose as inducer. *C. acetobutylicum* NCIB 8052, a strain that contains choline in the teichoic acids [15], was used to prepare [*methyl*-^{3}H]choline-labelled cell walls as previously described [4]. [*Methyl*-^{3}H]Choline- or [^{3}H]lysine-labelled cell walls of *S. pneumoniae* R6 were prepared according to the procedure described elsewhere [16]. Cell wall lytic activity was measured as the release of soluble radioactive marker from choline-containing cell walls [15,16]. The lytic activity on

unlabelled cells walls of *C. acetobutylicum* ATCC 824 was determined according to Croux et al. [1].

2.2. *DNA manipulations and DNA sequence analysis*

Plasmid DNA was prepared by the rapid alkaline method [12]. Restriction endonucleases, T4 DNA ligase and the Klenow fragment of the *E. coli* DNA polymerase were used according to the recommendations of the suppliers. *E. coli* cells were made competent by the rubidium chloride method [12]. DNA sequencing was performed by the dideoxy chain termination method [17] using a kit from Pharmacia.

2.3. *Maxicell and Western blot analysis*

Maxicell analysis was performed using *E. coli* MC4100 as previously described [4]. Western blot analyses were carried out using anti-pneumococcal LYTA amidase serum according to the procedure described elsewhere [10].

2.4. *Characterization of cell wall lytic activity*

The type of enzymatic activity (lysozyme or amidase) was determined by analysis on a Sephadex G-75 column of the degradation products resulting from the hydrolysis of choline-containing pneumococcal cells walls labelled either with [*methyl*-^{3}H]choline or [^{3}H]lysine [18,19].

3. RESULTS AND DISCUSSION

We had previously shown that the *Sna*BI restriction site of the *lytA* gene was located in the junction region between the N- and C-terminal domains of the LYTA amidase [8]. On the other hand, comparison of the amino acid sequence of the clostridial LYC enzyme with those of other lysozymes suggested that the *Bst*YI site of the *lyc* gene was also located in the junction zone between its corresponding N- and C-terminal domains. Hence, the chimeric gene, *lcl*, was constructed using both restriction sites according to the procedure described in Fig. 1. The expected in-frame ligation of both DNA fragments was confirmed by restriction enzyme and sequence analyses of the junction region. Competent cells of *E. coli* MC4100 were transformed with the recombinant plasmid, pLCL43, and maxicell analysis of the plasmid-encoded proteins showed the expression of the chimeric *lcl* gene under the control of the strong promoter, *lpp–lac*. Fig. 2 shows that *E. coli* MC4100 cells harbouring plasmid pLCL43 express a protein (LCL) with an apparent M_r of 35,000, which is in good agreement with the M_r of 34,000 deduced from the nucleotide sequence of the chimeric *lcl* gene. The other visible bands of M_r 30,000 and 40,000 correspond to the β-lactamase and the Lac repressor, respectively [4]. To confirm that the band of M_r 35,000 corresponds to the chimeric enzyme, an immunoblot analysis using an anti-LYTA serum was undertaken and revealed a major band of the predicted M_r in the sonicated extracts of *E. coli* DH1 (pLCL43) but not in the control extracts (Fig. 3). This band was also visible in the Coomassie brilliant blue-stained gel.

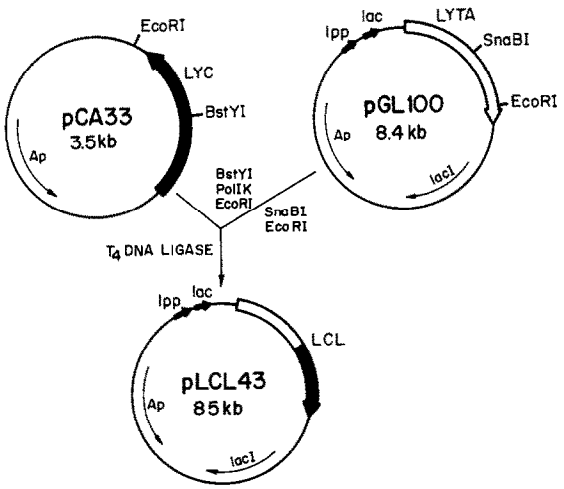

Fig. 1. Construction of the chimeric *lcl* gene. Plasmids are drawn in circles with the relevant elements and restriction sites indicated. Light line, vector plasmid; black box, structural sequence of the *lyc* gene; white box, structural sequence of the *lytA* gene. Arrows indicate the direction of transcription of the genes. Small arrows correspond to the lipoprotein promoter (*lpp*) and the Lac promoter–operator (*lac*). Ap, ampicillin resistance; *lacI*, Lac repressor gene; PolIk, Klenow fragment of the *E. coli* DNA polymerase.

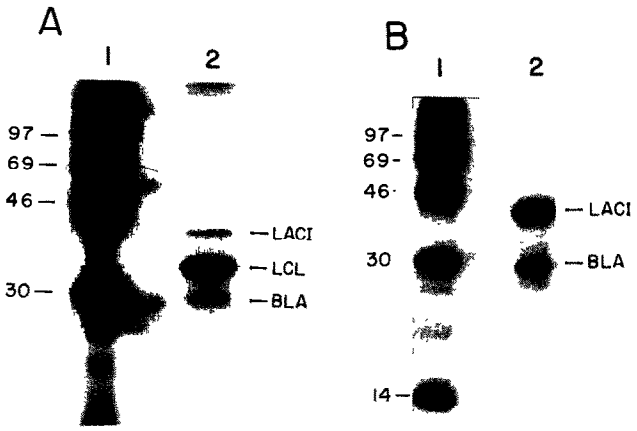

Fig. 2. Expression of the chimeric *lcl* gene in *E. coli* maxicells. Autoradiography of a 0.1% SDS 12.5% polyacrylamide gel showing the [^{35}S]methionine-labelled polypeptides synthesized in *E. coli* MC4100 (pLCL43) (lane 2, panel A) and *E. coli* MC4100 (pINII-A3) (lane 2, panel B). The molecular sizes of the [^{14}C]-labelled standard proteins (Amersham) are indicated on the left margin in kDa. Arrows show the positions of the chimeric LCL protein, the β-lactamase (BLA) and the lactose repressor protein (LACI).

To evaluate the enzymatic activity of the chimeric protein, sonicated extracts of *E. coli* DH1 (pLCL43) cultured in LB medium containing lactose were assayed using different cell wall substrates (Table I). The LCL protein was able to hydrolyze the cell walls of *C. acetobutylicum* NCIB 8052 and *S. pneumoniae* R6 but, interestingly, its optimal activity on either type of cell walls was achieved at different pH (Table I). Furthermore, the analysis of the fragments resulting from the digestion of the [^{3}H]choline-labelled pneumococcal cell walls with the chimeric LCL enzyme by chromatography on Sephadex G-75 columns indicated that this enzyme was an amidase (data not shown). This was an

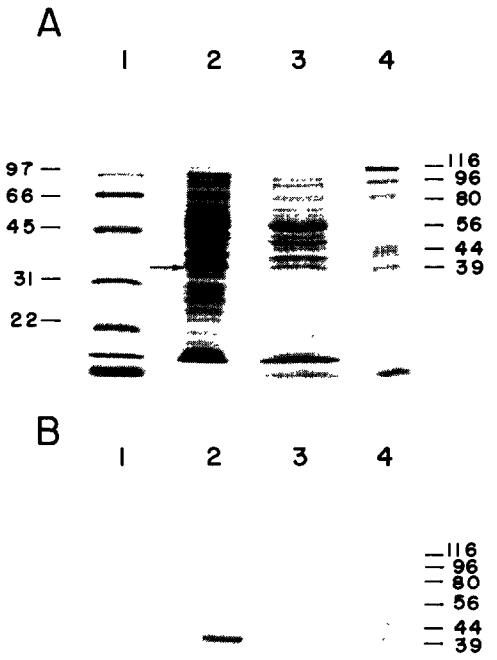

Fig. 3. Immunoblot analysis of the LCL protein. Sonicated samples were electrophoresed on a 0.1% SDS-12.5% polyacrylamide gel and stained with Coomassie brilliant blue (A) or developed with anti-LYTA serum (B). Lane 1, standard proteins (Bio-Rad); lane 2, total crude extract of *E. coli* DH1 (pLCL43); lane 3, total crude extract of *E. coli* DH1 (pINIII-A3); lane 4, Coomassie brilliant blue pre-stained standard proteins (Bio-Rad). The molecular sizes of the standard proteins are indicated in kDa. The arrow indicates the position of the LCL protein.

expected result since the chimera contains the LYTA amidase N-terminal domain which carries the catalytic center of this enzyme [11].

Comparison of the LCL activity with those of the N-LYTA amidase, the parental LYTA amidase and LYC lysozyme revealed several interesting features of this new enzyme. (Table I): (i) LCL did not degrade cell walls of *C. acetobutylicum* ATCC 824 under the conditions used; (ii) the chimeric LCL amidase turned out to be 250-times more active when assayed on cell walls of *C. acetobutylicum* NCIB 8052 than when tested on pneumococcal cell walls, in contrast to the behavior of LYTA and N-LYTA amidases; (iii) the optimal pH of the LCL amidase for the hydrolysis of the clostridial walls was identical to that reported for the LYC lysozyme but lower than the optimal pH for N-LYTA and LYTA amidases; (iv) LCL is a choline-independent enzyme since its activity was not inhibited by free choline. This last phenomena was to be expected since the C-terminal domain of the parental LYC lysozyme does not specifically recognize these residues.

The finding that LCL amidase more efficiently hydrolyzed clostridial cell walls than pneumococcal walls might be ascribed to a direct role of the C-terminal domain in substrate recognition due to the formation of a co-operative structure facilitated by the similarity in gene organization of the parental enzymes as already postulated [20]. We have previously suggested that the peculiar presence of choline in the pneumococcal cell wall has acted as an element of selective pressure preserving the C-terminal modules of most lytic enzyme of *S. pneumoniae* and its bacteriophages hence providing biological specificity to these enzymes and may also contribute to their improved catalytic efficiency [21]. The lysozymes of the fungus, *Chalara*, and the pneumococcal phage Cp-7, that possess C-terminal domains that do not have specific choline-recognition sites [7,22] and degrade pneumococcal cell walls in a manner independent of the presence of choline, as was also the case of the chimeric enzyme reported here, may represent examples in nature of the importance of a type of novel co-operativity between enzymatic domains to improve enzymatic activity. This type of co-operativity might be

Table I

Activity of the cell wall lytic enzymes

Enzyme	Type	Choline inhibition	Lytic activity on cell walls from[a]			
			C.a.824 (A)	C.a.8052 (B)	S.p. R6 (C)	(B/C)
LCL	Amidase	no	N.D.	1.3×10^6 (pH 5.0)	5.3×10^3 (pH 6.5)	245
LYTA	Amidase	yes	N.D.	0.3×10^3 (pH 6.5)	1.3×10^3 (pH 6.5)	0.2
LYC	Lysozyme	no	+ (pH 4.5)	3.7×10^4 (pH 5.0)	N.D.	–
N-LYTA	Amidase	no	N.D.	1.5×10^3 (pH 6.5)	2.0×10^3 (pH 6.5)	0.7

[a] Activity on cell walls of *C. acetobutylicum* NCIB 8052 (C.a.8052) or *S. pneumoniae* R6 (S.p.R6) is expressed as cpm released during 1 h of incubation at 37°C either per ml of crude extract in the case of LCL, LYC and N-LYTA or per ng of purified protein in the case of LYTA. Activity on cell walls of *C. acetobutylicum* ATCC 824 (C.a.824) was considered positive when the turbidity of the cell wall decreased after 1 h of incubation at 37°C. The optimal pH for enzyme activity is indicated. Choline inhibition was tested using 2% choline in the assay [8].
N.D., not detected.

the result of a role for this binding domain in the solubilization of the cell wall by modifying the conformation of the polymer to give the enzyme acces to the cleavable bonds or simply by increasing the local concentration of the substrate, as proposed for *Trichoderma reesei* cellulase [23]. On the other hand, it is well known that the composition of the peptidoglycan determines to some extent the specificity of cell wall lytic enzymes [2,24]. Hence, the finding that the chimera cannot hydrolyze the cell walls of the clostridial strain, ATCC 824, suggests that the composition and/or conformation of its peptidoglycan does not allow the correct orientation and/or stabilization of the muramoyl-peptide in the active center of this chimeric amidase.

We had observed that the C-terminal domains of the pneumococcal cell wall hydrolytic enzymes also play an important role as regards the optimal pH for enzyme activity [9]. The results presented in Table I fully confirmed this observation but indicate that the cell wall composition also influences the optimal pH. Since pH modifies the ionic interactions between the substrate and the C-terminal binding domain of these enzymes, and the anchoring of the enzyme to the cell wall strongly determines its catalytic efficiency, the pH might modulate the enzyme activity depending on the specific composition of either the cell walls or the binding C-terminal domains.

Finally, these results provide significant experimental support to the hypothesis that the LYC lysozyme has evolved by the fusion of two independent domains [3]. A noticeable novelty of the chimeric construction reported here, with respect to previous chimeric constructions, stems from the fact that we have fused two genes that lack sequence similarity and belong to different bacterial genera, thus confirming and extending our theory on the structural and functional relationships between pneumococcal and clostridial lysozymes [3,5,15]. Our results also strengthen the generally accepted view that one of the forces that drive the evolution of microorganisms is the potential of recombinant mechanisms for creating DNA fusions which allow them to adapt rapidly to new environments.

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