Rapid Method for Cloning a Ca^{2+}-Dependent Metalloprotease From a Gram-Positive Bacterium in *Escherichia coli*

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Cloning the genes of Ca^{2+}-dependent proteases from different genera of gram-positive bacteria such as *Bacillus* (1,10,11) or *Staphylococcus* (8) in *E. coli* is very often lethal for the recipient, and that is why these genes had to be cloned by propagating different flanks separately in *E. coli* or by choosing another cloning host (1,8–11). The *npr*M gene of *Bacillus megaterium* coding for the neutral protease also has a lethal effect on *E. coli* (9). However, by applying the mentioned strategies, it has been cloned and sequenced for different strains of *B. megaterium* (3,4). Here we describe a method that allows the cloning of this protease gene directly in *E. coli*. The method was developed for *B. megaterium* DSM 319, which is known to produce two extracellular proteases, a minor serine protease (9) and a neutral metalloprotease (5,6). It is central to the method and necessary that EDTA would complex the metal cofactor, eventually resulting in an enzymatically inactive protein.

We used the expression vector pUC*npr*M carrying, in addition to the ampicillin-resistance gene, the *npr*M gene governed by its own promoter (9). Cells of the strain *E. coli* HB101 were transformed following the Hanahan method (7), with the following modifications: a Ca^{2+}-free transformation buffer (10 mM 2-*N*-morpholinoethane sulfonic acid, pH 6.3, 45 mM $MnCl_2 \cdot 4 H_2O$, 10 mM $MgCl_2 \cdot 6 H_2O$, 100 mM KCl, 3 mM hexamine cobalt trichloride) was used. Furthermore, SOB-medium (2% Bacto-tryptone, 0.5% Bacto-yeast-extract, 10 mM NaCl, 2.5 mM KCl, pH 7.0) that was used to grow cells intended for an immediate transformation was made 20 mM in $MgCl_2$ (out of a 2 M $MgCl_2$ stock). To protect cells from neutral protease activity, transformations were performed in the presence of different concentrations of EDTA in the regeneration medium (liquid SOB medium without $MgCl_2$ with 20 mM glucose by adding a 1 M glucose stock) and/or the SOB plates (SOB medium solidified with 1.5% agar and containing 100 µg/mL ampicillin) on which transformants were selected. As SOB medium is <0.1 mM in Ca^{2+} and <0.5 mM in Mg^{2+} (2), which can both interact with EDTA, we tested concentrations ranging from 0 mM EDTA up to 0.5 mM EDTA (Figure 1). The use of a modified transformation buffer allows transformation of *E. coli* HB101 with pUC*npr*M, although transformation efficiency remains considerably low, i.e., 4 colony-forming units (cfu) per µg plasmid-DNA. Selection of transformants on SOB medium containing EDTA allowed increasing transformation efficiencies up to 18-fold (0.3 mM EDTA). Higher transformation efficiencies were achieved when the regeneration medium also contained EDTA. We obtained the best transformation results when using 0.1 mM EDTA in the regeneration medium and 0.3 mM EDTA in the selective medium (5.625×10^3 cfu/µg DNA; i.e., 140-fold higher when compared to a procedure without EDTA in regeneration and selective medium). In the regeneration medium, 0.1 mM EDTA proved to give the best results and should not be exceeded, as higher concentrations led to a decrease in transformation efficiencies (up to 6-fold, when compared to the highest transformation efficiency). Selective medium containing 0.4 or 0.5 mM EDTA also results in a decrease in transformation efficiencies (remaining below 10^3 cfu/µg DNA under all tested conditions), thus the concentration of EDTA in selective media should not exceed 0.3 mM.

Once the *npr*M-bearing plasmid is established in *E. coli*, cells can be propagated without addition of EDTA. Transformants grown on Ca^{2+}-caseinate agar (Merck, Darmstadt, Germany) containing 100 µg/mL ampicillin were found to form clear halos, indicating protease activity. Furthermore, the *B. megaterium* strain MS941, carrying an inactivated copy of the *npr*M gene (9), was transformed with pUC*npr*M isolated from transformed *E. coli* cells. The formation of clear halos on Ca^{2+}-caseinate agar by these transformants proved that pUC*npr*M from *E. coli* carries a functional copy of the *npr*M gene. This result was confirmed by the determination of protease activities (data to be published elsewhere). We suggest that the effect of the neutral

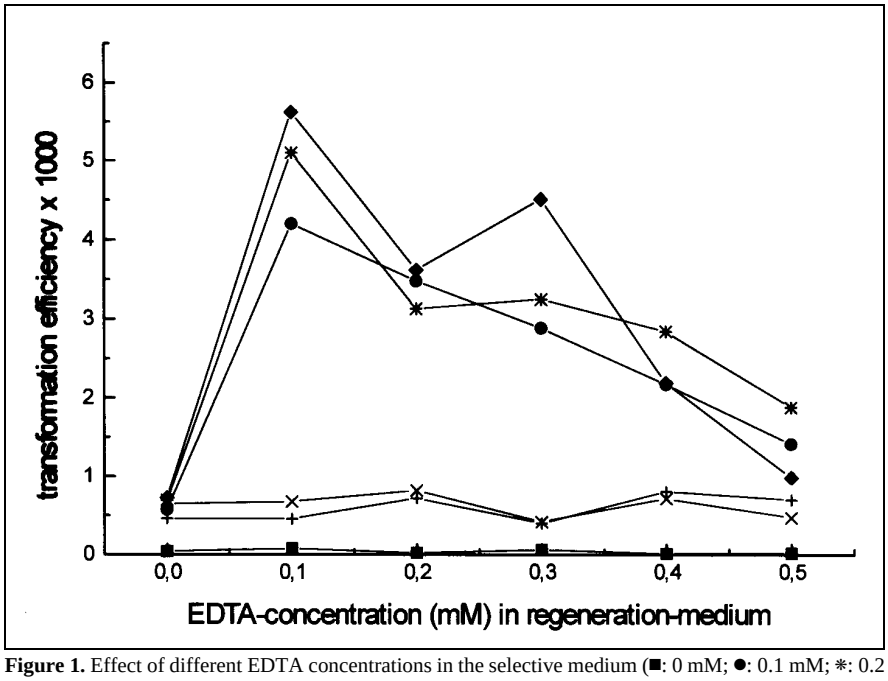

Figure 1. Effect of different EDTA concentrations in the selective medium (■: 0 mM; ●: 0.1 mM; ✳: 0.2 mM; ◆: 0.3 mM; +: 0.4 mM; ×: 0.5 mM) and the regeneration medium on the transformation efficiency, given as transformants per µg DNA, with data presented as means of triplicates.

protease in the early stages of transformation severely damages the cells. Hence, inhibition of the neutral protease would allow survival of the cells in this critical phase. The developed method might potentially apply to clone other genes encoding for Ca^{2+}-dependent metalloproteases in *E. coli*.

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