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Expression of the *Bacillus licheniformis* PWD-1 keratinase gene in *B. subtilis*

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The *kerA* gene which encodes the enzyme keratinase was isolated from the feather-degrading bacterium *Bacillus licheniformis* PWD-1. The entire gene, including pre-, pro- and mature protein regions, was cloned with P_{ker}, its own promoter, P43, the vegetative growth promoter, or the combination of P43-P_{ker} into plasmid pUB18. Transformation of the protease-deficient strain *B. subtilis* DB104 with these plasmids generated transformant strains FDB-3, FDB-108 and FDB-29 respectively. All transformants expressed active keratinase in both feather and LB media, in contrast to PWD-1, in which *kerA* was repressed when grown in LB medium. With P43-P_{ker} upstream of *kerA*, FDB-29 displayed the highest activity in feather medium. Production of keratinase in PWD-1 and transformants was further characterized when glucose or casamino acids were supplemented into the feather medium. These studies help understand the regulation of *kerA* expression and, in the long run, can help strain development and medium conditioning for the production of this industrially important keratinase.

Keywords: keratinase; gene cloning; gene expression; *Bacilli*

Introduction

The feather-degrading bacterium, *Bacillus licheniformis* PWD-1, its keratinase enzyme and gene *kerA*, have been isolated and characterized [8,9,14]. This bacterium and its enzyme are important for potential applications in animal feed production, waste treatment, food processing, detergents and pharmaceuticals. Previously, Northern analysis demonstrated that *kerA* was expressed inducibly in feather medium while soluble proteins had inhibitory effects on *kerA* expression [8]. These features restrict the use of various media for the growth of PWD-1 and production of this enzyme. Since *B. subtilis* and *B. licheniformis* are two closely related species, a protease-deficient strain *B. subtilis* DB104 was transformed by the *kerA* gene for overexpression. Methods of inserting *kerA* into plasmid vector pUB18 with different promoters for transformation, and subsequent characterization of keratinase production in *B. subtilis* transformants in various media are described in this report.

Materials and methods

Bacterial strains, plasmids and growth conditions

B. licheniformis PWD-1 (ATCC 53757) used in this study is a previously described patent strain isolated in this laboratory [12,14]. Plasmids pUB18 and pUB18-P43 were used for expression studies in *B. subtilis* DB104 (*his nprR*2 *nprE*18 *apr* Δ3) [7]. *Escherichia coli* INV αF′, and PCR-cloning vector pCRII were purchased from Invitrogen (Invitrogen Corp, San Diego, CA, USA). PCR-amplified 1.4-kb *kerA* fragment was previously inserted into the cloning site of pCRII, therefore the restriction sites flanking the cloning site facilitated the excision of the insert by double restriction digestion [8]. PWD-1 was grown on feather medium (per liter: NaCl, 0.5 g; $MgCl_2 \cdot 6H_2O$, 0.1 g; $CaCl_2$, 0.06 g; KH_2PO_4, 0.7 g; K_2HPO_4, 1.4 g; and chopped feathers, 10 g; pH 7.0), or Luria-Bertani (LB) medium at 50°C. *E. coli* INVαF′ was grown at 37°C on LB medium supplemented with ampicillin (50 μg ml^{-1}). *B. subtilis* DB104 was grown at 37°C in LB medium or feather medium. New transformants were cultured in feather or LB media with 20 μg ml^{-1} kanamycin. Tryptose blood agar base (TBAB) (Difco Laboratories, Detroit, MI, USA) plates containing 20 μg kanamycin ml^{-1} were used for routine transformations. A skim milk-feather plate (containing 5% Difco skim milk, 0.5% feather powder, 1% agar, and 20 μg kanamycin ml^{-1}) was used to screen for colonies producing keratinase.

DNA manipulations and vector constructions

Mini-preparation of plasmids harbored in *B. subtilis* DB104 were prepared by rapid alkaline sodium dodecyl sulfate method [10]. The 1.4-kb *kerA* fragment cloned in plasmid pCRII, was excised by *Xba*I and *Spe*I digestion [11]. Identical single-strand overhangs (5′-CTAG-3′) were generated at both ends (Figure 1a). Partial filling-in with nucleotides T and C created two-nucleotide overhangs (5′-CT-3′) at both ends of the insert. *B. subtilis* promoter P43 is a vegetative growth promoter which is activated by housekeeping sigma factor sA (sA = s43 = s55) [6,13]. Plasmid pUB18-P43 was created by inserting a DNA fragment (~300 bp) containing promoter P43 adjacent to the polycloning site of pUB18 [13]. When pUB18 and pUB18-P43 were digested by *Hind*III (5′-AAGCTT-3′), four-base over-

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hangs (5′-AGCT-3′) were generated at both ends. Partial fill-in with nucleotides A and G generated further two-nucleotide overhangs (5′-AG-3′) at the ends of the linearized vectors. These two separate treatments produced complementary overhangs on the vectors and insert, with their ligation yielding plasmids pLB3 and pLB29 (Figure 1a). As a result, the original *kerA* promoter, P_{ker}, and the combination of P_{ker}-P43 were presented on the upstream of the *kerA* coding region in plasmids pLB3 and pLB29, respectively.

To delete P_{ker} promoter from the 1.4-kb fragment, a PCR product was isolated by priming with the upstream oligonucleotide (5′-CCCTCTAGATTATTCTGAATAAAGAGG-3′, on the sense strand from position 183 to 201 of *kerA*, the *Xba*I site is underlined) and downstream primer (5′-GATCATGGAACGGATTC-3′, on the antisense strand from position 1457 to 1441 of *kerA*) (Figure 1b). A 182-bp upstream region containing P_{ker} was then deleted, resulting **kerA* (1.2 kb). This **kerA* was then inserted into plasmid pUB18-P43 at the *Sma*I site to yield plasmid pLB108 (Figure 1b).

Transformation of B. subtilis DB104 and screening for colonies harboring plasmid

Ligated DNA in 50 μl was added to 0.5 ml fresh-prepared *B. subtilis* DB104 competent cells [2,5]. After shaking at 150 rpm for 90 min, cells were plated on TBAB plates with 20 μg kanamycin ml^{-1}, and incubated at 37°C overnight. Colonies that grew on TBAB plates were then transferred to skim milk-feather plates for further screening. Colonies with clear haloes resulting from proteolysis were selected for plasmid isolation and keratinolytic activity analysis. DB104/pUB18 and DB104/pUB18-P43 cells were used as negative controls for screening of *kerA*-containing colonies. Plasmids isolated from halo-forming transformants of DB104 were then characterized by restriction digestion and PCR analysis.

Determination of kerA expression in B. subtilis

B. licheniformis PWD-1, and *B. subtilis* DB104 transformants were grown in LB and feather media to examine the expression of *kerA*. Samples were taken every 4 h from LB culture medium and every 12 h from feather culture medium, and their keratinolytic activity determined by azo-keratin hydrolysis [9]. To determine the effects of glucose and soluble peptides and amino acids on *kerA* expression, 0.5% of glucose (w/v) or 0.5% casamino acid (w/v) was supplemented into feather medium.

Results and discussion

*Plasmids harboring kerA or *kerA*

Plasmids pLB3 and pLB29 were created by inserting *kerA* into pUB18 and pUB18-P43, respectively (Figure 1a). Linearized pLB3 and pLB29 (digested by *Xba*I) displayed a 1.4-kb increase in size on 1.0% agarose gel as expected (data not shown). When these plasmids were used as templates for PCR amplification, 1.4-kb fragments were produced in the reaction with *kerA*-specific primers. Thus, pLB3 harbors only the *kerA* promoter, P_{ker}, whereas pLB29 contains P_{ker}-P43 oriented for the transcription of keratinase. Plasmid pLB108 was constructed by inserting the 1.2-kb **kerA* without P_{ker} into pUB18. The insertion was confirmed by restriction digestion and PCR analysis. All three plasmids were used to transform the protease-deficient *B. subtilis* strain DB104.

Expression of kerA in B. subtilis

Wild type PWD-1, DB104/pUB18 control, FDB-3 (DB104/pLB3), FDB29 (DB104/pLB29) and FDB108-(DB104/pLB108) grew rapidly in LB medium. Data from azo-keratin assays indicated that all new transformants were capable of producing keratinase in LB medium (Figure 2). Neither PWD-1 nor the DB104/pUB18 control produced keratinase in LB medium. In feather medium, FDB-3, -29 and -108 and PWD-1 all produced keratinase, but FDB-29 yielded the highest activity (3-fold higher than PWD-1) among the three transformants (Figure 2). The two promoters, P43 and P_{ker} in FDB-29 seem to have an additive effect on each other.

Immunoprecipitation assays and SDS-polyacrylamide gel electrophoresis (data not shown) analyses of both LB and feather media confirmed the production of keratinase by the transformants.

It appears that the P43 promoter enhanced the expression of *kerA* (Figure 2). Rapid growth of FDB-29 cells along with increasing keratinolytic activity was observed in feather medium (Figure 2). On the other hand, FDB-3, which contains the plasmid pLB3 lacking the P43 promoter, showed a long adaptive period and produced a lower level of keratinase than PWD-1 grown in feather medium. It is believed that FDB-3 requires an induction process for the expression of *kerA*. FDB108, which possesses the P43 promoter, but without P_{ker}, on its plasmid pLB108, showed increased keratinase production (Figure 2). Evidently, harboring plasmids with *kerA* enables all three transformants to hydrolyze and grow on feathers. On the other hand, the DB104/pUB18 control grew poorly in feather medium and displayed no feather hydrolysis. These results confirmed that *kerA*, previously isolated from *B. licheniformis* PWD-1 [8], is indeed the gene encoding the keratinolytic enzyme.

In the soluble protein-rich LB medium, FDB-3, -29 and -108 all grew well and produced significant levels of keratinase, whereas PWD-1 produced little keratinolytic activity. These data agree with previous results of Northern analysis for mRNA in PWD-1 [8]. The phenomenon of catabolite repression, by which readily metabolizable carbon sources suppress the use of less readily metabolizable substrates, was obvious for PWD-1 but not found for FDB-3, -29 or -108 in LB medium. This may indicate that the site for repression initiated by the presence of soluble proteins was not present within the 200-bp *kerA* promoter region, nor in the P43 promoter region.

Effects of glucose and casamino acid on kerA expression in feather medium

When 0.5% glucose was added to feather medium, keratinase production by PWD-1 was completely repressed (Figure 3). Supplementation of glucose in FDB-29 decreased *kerA* expression to a level comparable to that in FDB-3. FDB-3 grown in feather medium was insensitive to addition of glucose. FDB-108, the transformant without

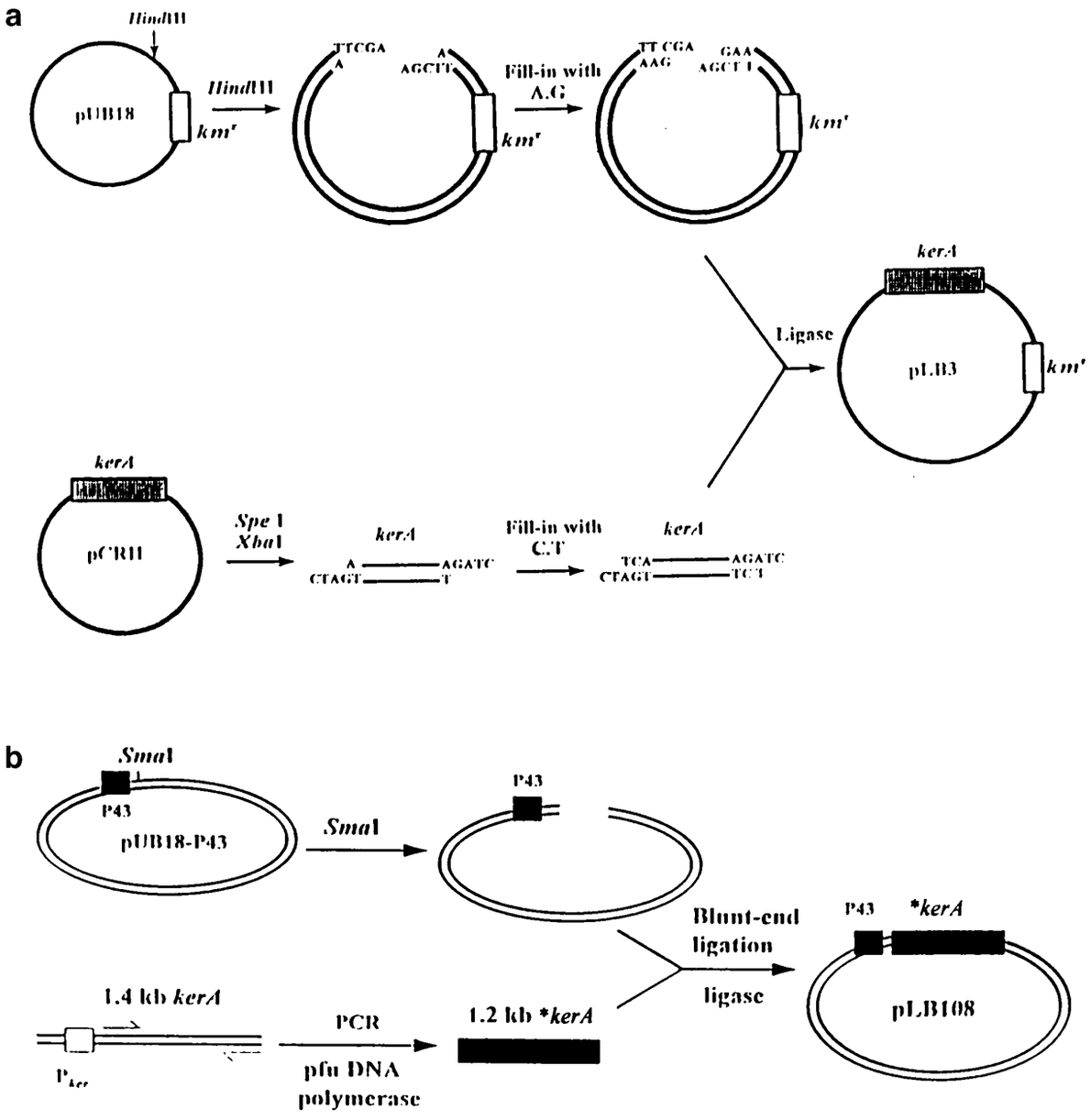

Figure 1 Construction of plasmid vectors. (a) Plasmid pLB3. pLB29 was constructed the same way, except that pUB-P43 as in (b) was used. (b) Plasmid pLB108. *kerA* is the intact 1.4 kb and **kerA* is the 200-bp shortened *kerA* (P_{ker} deleted).

P_{ker}, demonstrated an elevated production of keratinase in feather-glucose medium. Glucose is known as a common catabolic repressor for a number of enzymes in *B. subtilis* [1,4]. Since glucose could lower the keratinase in FDB-29 to a level similar to that of FDB-3 in feather medium, P43 is believed to be down-regulated by glucose in the combined P43-P_{ker} system.

Casamino acids, like glucose, also repressed the expression of *kerA* in PWD-1 in feather medium, but had little effect on FDB-3 and slight increase for enzyme production in FDB-29. In FDB-108, a 100% increase in keratinase production was observed in feather-casamino acid medium and an approximately 50% increase in feather-glucose medium (Figure 3). The reason for the stimulating effects of amino acids and glucose on FDB-108 which contains only P43 is not known. Perhaps P43 responds positively to a rich medium for vegetative growth. Since FDB-3 cultures were inert to both casamino acids and glucose supplementations, the stimulating effects from glucose and casamino acids in FDB108 might be due to the absence of glucose and amino acid repression sites in the 200-bp upstream region in the 1.4-kb *kerA* fragment.

In this study, *B. subtilis* strain DB104, which is deficient in neutral and alkaline proteases [7], was tested as the host cell for the cloning and expression of *kerA*. Our results indicate that DB104 was able to express *kerA* originated from *B. licheniformis* PWD-1 and secrete active keratinase into the medium at a high level. Furthermore, DB104 transformants with different promoter systems can serve as models to study the effects of glucose and amino acids on the expression of *kerA*.

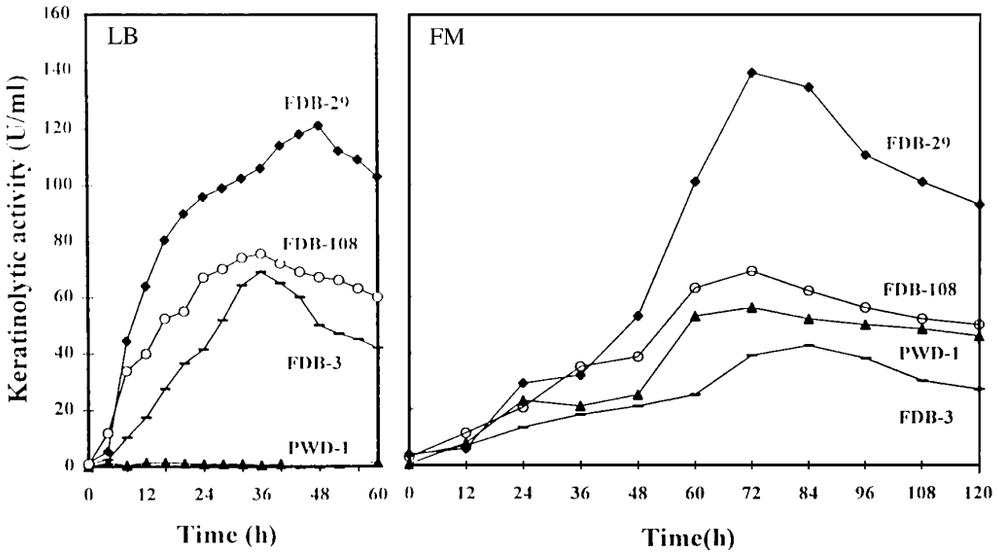

Figure 2 Expression of *kerA* in LB medium and feather medium (FM). Keratinolytic activity was measured by azokeratin hydrolysis [10]. One unit of keratinolytic activity was defined as an increase of A_{450} by 0.01 after reaction with azo-keratin for 30 min.

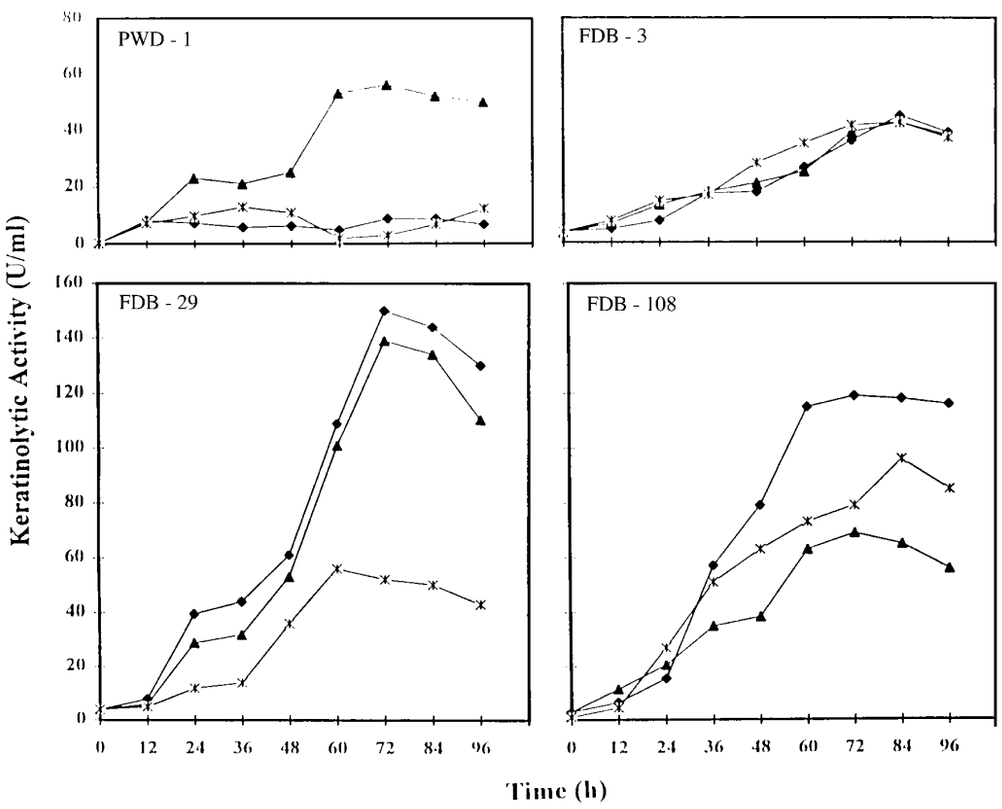

Figure 3 Effects of glucose and casamino acid on *kerA* expression as measured by the keratinolytic activity in feather (▲), feather-glucose (✳) and feather-casamino acid (◆) media.

The pre- and pro-regions of *kerA* were apparently recognized and processed in DB104. A similar *B. subtilis* strain WB600, which is deficient in six proteases [15], was also tested for *kerA* expression in pLB29, but low keratinase activity was produced in LB medium. For effective production of a foreign protein, it is generally believed that high levels of proteases in the host cell may be detrimental, though a low level of proteolytic enzymes may be necessary for processing and maturation [3]. In FDB-29, it seems that all the elements including the host cell DB104, P43 promoter, pre- and pro-regions of *kerA*, performed compatibly and efficiently for keratinase production.

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