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THE INFLUENCE OF *Bacillus subtilis* PROTEINS DegU, SinR AND SinIR ON BACITRACIN BIOSYNTHESIS IN *Bacillus licheniformis*

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A B S T R A C T

A vast array of natural peptides diverse in their structure that are produced by microorganisms living in different aquatic and terrestrial environments are not gene encoded but are synthetized nonribosomally on peptide synthetases. The branched cyclic dodecyl peptide antibiotic bacitracin, produced by special strains of *Bacillus,* is synthesized nonribosomally by a large multienzyme complex composed of the three bacitracin synthetases BA1, BA2 and BA3. The rate of peptide antibiotic bacitracin biosynthesis positively correlates with the level of the alkaline protease production. To define some positively acting factors in terms of bacitracin production we performed an experiment expressing genes involved in the process of sporulation and competence (*B. subtilis*): *degU* on pBD1834, *sinIR* on pIS74 and *sinR* on pIS119 in *B. licheniformis*. Bacterial clones were analyzed for the proteolytic activity and bacitracin production. In 9 clones (2 x pBD1834, 2 x pIS74 in 5 x pIS119) higher rate of the peptide antibiotic bacitracin production was determined.

Key words: microbiology / bacteria / *Bacillus* / antibiotics / bacitracin / biosynthesis

VPLIV BELJAKOVIN DegU, SinR IN SinIR BAKTERIJE *Bacillus subtilis* NA BIOSINTEZO BACITRACINA PRI BAKTERIJI *Bacillus licheniformis*

I Z V L E Č E K

Mikroorganizmi iz različnih okolij, od vodnih do talnih, proizvajajo številne naravne peptide z izjemno strukturno raznolikostjo. Le-teh ne kodirajo geni. Njihova biosinteza poteka neribosomsko na velikih multifunkcionalnih encimih, peptidnih sintetazah. Bacitracin je razvejan ciklični dodecilpeptidni antibiotik. Proizvajajo ga nekateri sevi rodu *Bacillus* s pomočjo velikega multiencimskega kompleksa, zgrajenega iz treh sintetaz bacitracina: BA1, BA2 in BA3, "neribosomsko". Raven biosinteze peptidnega antibiotika bacitracina pozitivno korelira z ravnijo proizvodnje alkalne proteinaze. Da bi določili nekaj dejavnikov s pozitivnim delovanjem glede na biosintezo bacitracina, smo izvedli poskus z izražanjem genov, ki sodelujejo v procesih sporulacije in kompetence *(B. subtilis): degU* v pBD1834, *sinIR* v pIS74 in *sinR* v pIS119, v bakteriji *B. licheniformis*. Klone bakterij smo nato analizirali. Pri 9 klonih (2 x pBD1834, 2 x pIS74 in 5 x pIS119) smo ugotovili višjo raven proizvodnje peptidnega antibiotika bacitracina.

Ključne besede: mikrobiologija / bakterije / *Bacillus* / antibiotiki / bacitracin / biosinteza

INTRODUCTION

Many natural peptides with remarkable structural diversity produced by microorganisms living in different habitats, spread from aquatic to terrestrial environments, are not gene encoded but are synthesised nonribosomally on large multifunctional enzymes called peptide synthetases. In this nonribosomal mechanism of peptide synthesis, compounds such as lipopeptides, depsipeptides, and peptidolactones are assembled from an exceedingly diverse group of precursors (more than 300 known) including pseudo, nonproteinogenic, hydroxy, *N*-methylated, and D-amino acids. The peptide backbone of these bioactive peptides can be composed of linear, cyclic, or cyclic branched structures (bacitracin - *Bacillus licheniformis*) that can be further modified by acylation, glycosylation, or heterocyclic ring formation. Some express antimicrobial, antiviral or antitumor activities. Others express immunosupressive or enzyme inhibiting activity (Marahiel *et al.*, 1997). These peptides are used in medicine and in agriculture. They are structurally diverse but they have the same mode of biosynthesis through the multienzyme thiotemplate mechanism. Peptide bonds are formed on or by multinenzymes - peptide synthetases. Amino acids are first activated to the corresponding adenylates (unstable intermediates) by the ATP hydrolysis. Adenylate is subsequently transferred to another site of the multienzyme molecule where it is bound as a thioester to the cysteamine group of an enzyme bound 4'- phosphopantetheinyl (4'- PP) cofactor. Thioesterified substrate amino acids are then integrated into the peptide product through exact (enzymaticaly determined) number of elongation steps - transpeptidation reactions. Transpeptidation reactions occur by transfer of the thioester-activated carboxyl group of one residue to the adjacent amino group of the next amino acid (N to C stepwise assembly of the peptide product). During the condensation process all intermediates are covalently attached to the multienzyme complex.

Bacitracins are antibiotic polypeptides produced by certain strains of *B. licheniformis* and *B. subtilis*. Crudely purified bacitracin contains at least 10 distinct dodecapeptides that differ by one or two aminoacids. The most abundant and best characterized of these peptides is bacitracin A, a branched cyclic dodecapeptide. It contains an amino-terminal pentapeptide moiety with an isoleucine-cysteine thiazoline condenzation product and a carboxy-terminal heptapeptide ring, in which the free α-carboxy group of the carboxy-terminal Asn is bound to the ε-amino group of lysine. As well as proteinogenic amino acids, four amino acids in the D-configuration (Glu4, Orn7, Phe9 and Asp11) and the nonproteinogenic residue ornithine (Orn) are incorporated into bacitracin A. Bacitracin A is most active against Gram-positive bacteria and acts by inhibiting bacterial cell wall biosynthesis: bacitracin A forms a tight ternary complex with C_{55}-isoprenyl pyrophosphate (IPP) and a divalent metal cation. IPP serves as a membrane-associated carrier during the synthesis of the repeat subunits of peptidoglycan. Recycling of IPP involves its dephosphorilation to C_{55}-isoprenyl phosphate (IP) by a phosphatase at the end of each cycle; this step is blocked by the bacitracin-M^{2+}-IPP-complex (where M^{2+} is a divalent metal ion), resulting in an accumulation of uridine diphosphate-acetymuramyl pentapeptide. Besides this primary mode of action, bacitracin also seems to affect membrane functions, the action of certain hydrolytic enzymes, and the biosynthesis of ubiquinone precursors. The genes encoding the three bacitracin synthetases (BA1, BA2 and BA3) were found to be organized in an operon. BA1 is encoded by *bacA*, the first gene of the operon. It activates and incorporates five amino-terminal aminoacids (isoleucine + cysteine forming the thiazoline ring, leucine, D-glutamine and isoleucine). BA2 is encoded by *bacB*, and is responsible for the activation and incorporation of two amino acids (lysine-D-ornithine). BA3 (*bacC*) is responsible for incorporating five amino acids (isoleucine-D-phenylalanine-histidine-D-aspartate-asparagine) into bacitracin.

In our strain improvement program we started testing the influence of increased concentration of polypeptides involved in regulation of the late log-phase to stationary phase

events. DNA fragment codding the thioesterase - enzyme responsible for elimination of bacitracin from the synthetase molecule was isolated as well. For identification and cloning peptide synthetase genes a general approach evolved by Turgay and Marahiel (1994) was used: PCR with degenerated oligonucleotides (primers) derived from the highly conserved core sequences TGD and LGGXS of the peptide synthetase was successfully performed.

B. licheniformis produces numerous degradative enzymes, including levansucrase, α-amylase, and proteases. The expression level of secreted proteins is globally controlled by the products of at least three genes: *degQ, degS,* and *degU* (Klier *et al.*, 1992). *degU* was originally identified because it is needed for the expression of certain degradative enzymes, mostly extracellular that are normally synthesised at an enhanced rate postexponentially (Dubnau, 1991). The *degS* and *degU* form an operon encoding a two-component system. The DegS (histidine kinase) protein is probably a cytoplasmic protein, but it does not contain hydrophobic domains required for insertion into membrane. It is supposed that DegU effector could exist under two active conformations :

- the phosphorylated form would be required for degradative enzyme synthesis while,
- the unphosphorylated form would be necessary for the expression of competence genes.

Both forms of DegU are required for two distinct functions and apparently act as positive regulators. Dependence of expression of levansucrase in *degU* is bypassed mutations in *mecA* and *mecB*. This implies that DegU may not interact directly with targets near the degradative enzyme promoters and suggests that *mecA* gene product plays a central role in this regulatory network. The expression of *degQ* is subject to growth-phase regulation and was reduced in a strain deleted for the *degS-degU* genes. DegQ protein in higher concentrations (overexpressed) stimulates the alkaline protease production (AprE). The rate of AprE production in *B. licheniformis* BA1 strain positively correlates with bacitracin production. We studied *B. licheniformis* BA1 transformants (pBD1834) expressing the *degU* gene in multiple copies. Since expression of *degQ* is controlled by both the DegS-DegU and ComP-ComA two-component systems, we expected certain positive or negative shift in bacitracin biosynthesis capacity of the pBD1834 transformants. *comA* and *comP* are adjacent genes. On the basis of their predicted amino sequences, they appear to encode response regulator and histidine kinase proteins, respectively. In addition to the resemblance of the ComA N-terminal domain, which contains three highly conserved aspartic acid residues which form a putative phosphorylation site (Klier *et al.*, 1992), to other response regulators, the C-terminal sequences of several known DNA-binding proteins and to DegU. These similarities, plus the effects of *comA* and *degU* mutations on expression of a number of genes, imply that the two response regulators function as transcription factors (Dubnau, 1991). The sensor kinase ComP promotes the establishment of competence. The stimulus detected by the membrane receptor domain could be the level of a crucial nutrient. Just upstream from the *comP* and *comA* genes lies the *comQ* gene, whose product is thought to act together with the ComP-ComA regulators to positively control expression of *srfA* operon , which on turn leads to expression of *comK* and of late competence genes (Klier *et al.*, 1992).

Similar shifts concerning bacitracin production were expected in transformants of *B. licheniformis* BA1 transformed with plasmids carrying *sinR* and *sinIR*. SinR protein represses sporulation, therefore its inactivation (with SinI) causes loss of competence and motility. It is required of the expression of the late competence genes and motility genes as well as for autolysin production. Presence of *sin* gene on a multicopy plasmid reduces expression of *spoIIA, spoIIG, and spoIIF.* Moreover, expression of the *spoIIE* gene is slightly higher when the *sin* gene is disrupted. Sin can control target genes transcribed by RNA polymerase with different σ factors (Klier *et al.*, 1992).

MATERIAL AND METHODS

Strains: *Bacillus licheniformis* BA1 (KRKA - production strain), *B. subtilis*.

Plasmids (donated by Prof. I. Smith for the research purpose only): pBD1834 containing *degU*, pIS74 containing s*inIR,* pIS119 containing *sinR* . For the fast estimation of the bacitracin concentration the TLC method was used (Merck HPTLC 13728, mobile phase: n-buthanol : acetic acid : H_2O = 4 : 1 : 2). For identification and cloning of peptide synthetase genes general approach evolved by Turgay and Marahiel (1994) was used: PCR with degenerated oligonucleotides (primers) derived from the highly conserved core sequences TGD and LGGXS of the peptide synthetase was successfully performed.

For the physiology experiments laboratory 3 l bioreactors (B. Braun Biotech Biostat MD) containing 1.5 l of the production medium composed of soy flour, starch and minerals or mineral medium made up in 20 l batches with glucose (0.2 to 1%) were used. Chemostat cultivated (36°C, 900 rpm, mineral medium with 0.2 to 1% of glucose) microbial population was checked for the production of bacitracin in correlation to the production of the late growth phase enzymes: alkaline protease (azocasein method) and alkaline phosphatase (p-nitrophenyl phosphate as a substrate).

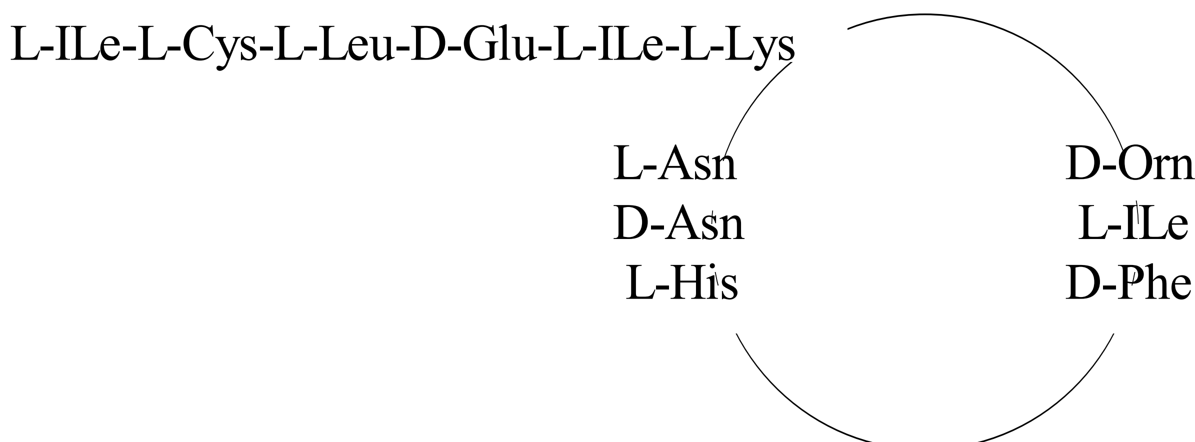

Figure 1. Bacitracin
Slika 1. Bacitracin

RESULTS AND DISCUSSION

Bacillus *licheniformis* strain BA1, transformed with pBD1834, pIS74 and pIS119 produced up to 100% more bacitracin compared to the standard production strain. The rate of bacitracin production strongly correlated with the rate of alkaline proteinase activity.

Due to homology of *B. licheniformis* and *B. subtitles* physiology we expected similar behaviour of pIS74, pIS119 and pBD1834 transformed *B. licheniformis* cells concerning sporulation events. *B. subtilis* (strains 119, 120, 510 and 1012) pIS74 transformants sporulated at

least in 90%. *B. subtilis* (strains 119, 120, 510 and 1012) pIS119 transformants sporulated in less than 50%. DNA binding protein SinR required for competence, inhibits sporulation in *B. subtilis.* We observed 100% inhibition of sporulation in *B. licheniformis* pIS119 transformants. When two genes *sinI* and *sinR* were expressed simultaneously 20 – 60% of pIS74 transformed *B. licheniformis* BA1 cells sporulated after 42 hour incubation in sporulation medium. Cell morphology turned from thicker to thinner, longer and immobile rods in 22 hour. In 42 hours in pIS74 and pIS119 transformants unsporulated cells turned to shorter and mobile rods.

Table1. Biosynthetic potential of recombinant *Bacillus licheniformis* strains [1]
Preglednica1. Biosintezni potencial rekombinantnih sevov *Bacillus licheniformis* BA1 [1]

	Protein Concentration (%) Koncentracija Beljakovin (%)	Alkaline proteinase activity Aktivnost alkalne proteinaze	Alkaline proteinase g^{-1} protein activity Aktivnost alkalne proteinaze g^{-1} beljakovin	Bacitracin content (%) Vsebnost bacitracina (%)	Bacitracin content g^{-1} Vsebnost Bacitracina g^{-1}
B. licheniformis BA1	100	100	100	100	100
B. licheniformis BA1 pIS74 1	94.20 98.27 95.47	93.57 94.63 95.06	100.00 96.46 100.00	96.36 110.92 104.47	102.21 112.79 109.34
B. licheniformis BA1 pIS74 5	90.77 87.63 85.46	72.56 80.63 74.90	80.38 92.18 87.10	87.29 90.18 97.29	96.10 102.81 112.43
B. licheniformis BA1 pIS119 1	103.87 95.47 81.45	95.82 77.81 110.10	89.71 97.10 132.15	137.12 137.88 132.84	128.04 140.07 158.19
B. licheniformis BA1 pIS119 2	87.50 90.47 76.97	110.44 151.44 139.69	122.50 163.02 176.52	165.79 146.11 157.66	183.76 156.63 198.65
B. licheniformis BA1 pIS119 3	87.42 97.63 95.21	181.20 145.69 141.78	201.60 144.69 144.69	148.94 141.19 147.93	165.24 140.25 150.38
B. licheniformis BA1 pIS119 4	94.47 93.87 61.18	156.40 167.89 56.92	161.88 173.63 90.67	160.00 199.47 15.38	164.25 206.09 24.37
B. licheniformis BA1 pIS119 5	94.16 67.18 80.39	151.96 173.37 158.22	157.23 250.80 190.99	131.19 106.90 123.59	135.13 154.31 149.09
B. licheniformis BA1 pBD1834 4	117.77 119.40 121.66	114.03 117.09 116.37	97.45 97.45 96.75	144.43 135.74 130.08	122.77 113.81 107.04
B. licheniformis BA1 pBD1834 5	118.70 117.72 116.76	111.51 122.84 135.61	93.97 104.41 116.24	126.65 138.46 138.94	106.81 117.75 119.13

[1] Results in Table 1 are expressed as relative values in % concerning the original strain *B. licheniformis* BA1.
[1] Rezultati v preglednici 1 so podani v relativnih vrednostih v % glede na izhodni sev *B. licheniformis* BA1.

The results are contradictory. With the retransformation of *B. subtilis* we proved that we successfully transformed protoplasts of *B. licheniformis* with pIS74 and pIS119 (carrying *sinIR* and *sinR* respectively). What we do not know is whether these genes are expressed in the

recombinant cells. In the case where the cells were transformed with *sinIR* we could not be sure whether both genes were expressed or just one. At this stage a reporter gene should be introduced to enable us to trace the expression of the regulatory genes under examination.

It is possible that a complete or partial recombination between the plasmid and chromosomal DNA took place in the recombinatory cell.

Further studies are needed to clarify the function of SinR and SinIR in the process of competence and to understand the mechanism of the regulation itself. The question here is whether the mechanism of regulation resembles that of *B. subtilis*. We also need to examine whether the role of the regulatory proteins is the same and whether their target locations coincide with those in *B. subtilis.*

There is some evidence that bacitracin prolongs the process of competence, but it remains to be established to what degree it affects the competence and sporulation processes and how it affects the Sin proteins.

In our case it is also unclear how many generations retain their recombination stability.

In pBD1834 (*degU*) transformed cells of *B. subtilis* and *B. licheniformis* no cells sporulated as expected. After 22 and 42 hour incubation in sporulation medium cellular morphology remained more or less constant – motile rods.

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