Extracellular polyhydroxybutyrate depolymerase/esterase of *T. thermophilus* is associated with flagellin of the type III export system

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Abstract

Extracellular polyhydroxybutyrate (PHB) depolymerase/esterase activities were isolated from the T. thermophilus culture medium, and they were found to be associated strongly with a protein of approximately 32.6 kDa, the flagellin of the type III export system. This identification was performed by N-terminal and internal sequence analysis

Introduction

T. thermophilus and related species are frequently used as hosts for the industrial production of thermophilic and thermostable proteins. The easy cultivation and the high natural capacity to secrete proteins into the growth medium qualify *T. thermophilus* as an industrial host for the overproduction of homologous and recombinant proteins. However, the literature on thermophilic PHA-degrading bacteria and thermostable PHA depolymerases is limited.

Bacteria secrete small compounds or bulky proteins, including substances used for the construction of extracellular organelles like flagella. The export systems that are required to guide proteins through the inner and outer membranes are derived from complicated systems of multiple genes. Recently, type III export systems have been shown to be responsible for flagellar biogenesis and for the export of virulence factors (Pugsley 1993). Bacterial flagella contain flagellins (Fla) with conserved N-and C-terminal domains, but greatly divergent internal regions, which are essential for export and polymerization of the Fla monomers, respectively (Homma et al., 1987). The complex flagellar structure is composed of a basal body, hook and filament; the latter accounting for 98% of the organelle mass and composed exclusively of Fla, the product of the *hag* gene. The basal structure is engaged in protein secretion, before serving as the flagellar motor afterward. Although the export mechanism for Fla has not been elucidated, it does not seem to be directed by a posttranslationally processed N-terminal signal peptide.

In response to any environment that no longer supports growth and proliferation, including nutrient deprivation, bacteria move away from adverse conditions using their flagellar organelle. Specific peptides secreted into the culture medium may serve as signals for high-density cultures and have been implicated in quorum sensing (Fuqua et al.,1996; Kleerebezem et al., 1997). Cells exhibit hypermotility *via* both increased numbers of motile cells and increased movement, as they reach the end of the exponential growth phase upon nutrient exhaustion (Nishihara and Freese, 1975). In this phase, the increased Fla expression may be triggered by nutrient deprivation, high cell density, and/or the initiation of transition state phenomena (Strauch 1993).

The polyhydroxyalkanoate (PHA)-degrading microorganisms secrete extracellular PHA depolymerases, which degrade PHA and utilize the products as nutrients. Extracellular degradation occurs when a not-necessary-accumulating microorganism utilizes exogenous carbon sources or PHAs released after lysis of accumulating bacteria. This ability for PHA degradation is due to the secretion of specific PHA depolymerases that are carboxyesterases (EC 3.1.1) that hydrolyze the water-insoluble polymer to water-soluble oligomers or monomers (Jendrossek et al., 1993).

In this work the product of the *hag* gene, flagellin, was isolated from the culture medium of *T. thermophilus* grown in minimal medium containing sodium gluconate as carbon source, and found to be strongly associated with the extracellular PHB depolymerase/esterase.

Materials and methods

Bacterial strain and cultivation conditions. T. thermophilus HB8 (DSM 579) was used. Polymer-degrading bacteria were grown in mineral medium containing sodium gluconate or PHB as carbon sources.

PHA depolymerase assays. PHA depolymerase activities were assayed using as *p*-nitrophenyl butyrate (PNPB) or PHB as substrate (Jendrossek et al., 1993).

Protein characterization: N-terminal sequence analysis. Protein samples were subjected to SDS-PAGE, and the corresponding 32.6kDa band was visualized with Coomassie Brillant Blue and subjected to Edman degradation. Automated N-terminal sequence analysis was performed on an Applied Biosystems protein sequencer (492A) applying pulsed liquid blot mode chemistry. The deduced N-terminal sequence was analyzed using SwissProt protein sequence data.

Matrix-assisted laser desorption mass spectrometry (MALDI-MS). Protein samples were subjected to SDS-PAGE. The 32.6kDa band was excised from the gel and subjected to MALDI-MS (Bruker Reflex III). Following digestion with 400 ng trypsin (Roche Biochemicals), the resulting peptides were desalted using ZIP-TIPS (Millipore) and spotted onto Bruker gold anchor (AnchorChip 400) targets with DHB as a matrix. Analysis was carried out in positive mode with a mass range of 500-3500Da. Data analysis was performed using Biotools software and Mascot search algorithms.

Results

Purification of PHB depolymerases. The crude PHB depolymerase extract was obtained by centrifuging the culture medium and removing the cells. The 6000xg supernate was concentrated by passage through a ultrafiltration nitrocellulose membrane (Amicon) with 10 kDa-cutoff and applied on a DEAE-cellulose, an affinity chromatography of silica-PHB and Sephadex G-200 columns. Two peaks of PHB depolymerase activity were separated from the last Sephadex G-200 column. One peak which was eluted in the void volume of the column exhibiting a high molecular weight aggregate and another peak approximately at molecular weight of bovine serum albumin (Fig. 1, lanes 1,2). The high molecular weight aggregate was analyzed on SDS-PAGE after heating at 100^{o}C for 15 min. As we can see at Fig. 1 (lane 1), a major protein band of 32.6kDa appeared. The two faint bands of molecular weight approximately 90-kDa and 67-kDa, presented PHB depolymerase/esterase activity. Therefore, it was strongly indicated by this experiment that these three bands are formed a dissociable complex.

Further purification and zymogram analysis of 90-kDa and 67-kDa protein bands showed that both proteins exhibited PHB depolymerase/esterase activity (Fig. 1, lanes 2, 3). The 32.6kDa protein was further characterized by N-terminal sequence, MALDI-MS analysis and homology investigation as flagellin.

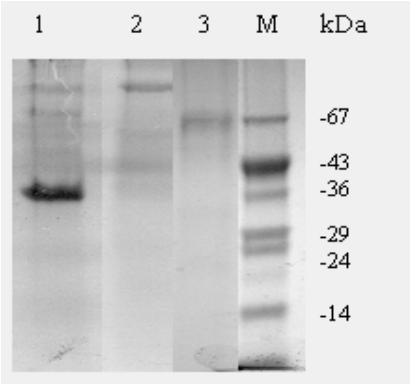

Fig 1: SDS electrophoresis of extracellular PHB depolymerases from *T. thermophilus*.
Lanes 1: PHB depolymerase-flagellin complex from Sephadex G-200 chromatography. *Lanes 2 and 3:* Purified PHB depolymerases of 90-kDa and 67-kDa, respectively. *Lane M:* molecular weight markers.

The sequence of the first 15 amino acid residues determined for the purified 32.6-kDa protein of *T. thermophilus* is: [1]MR**INHNIAAL NTLNR** [15]--, where the first two residues are missing. A NCBI databank search located similarities of this N-terminal sequence with flagellin from *Thermotoga maritima* MSB8 (NP_228567.1; AAD35840) possessing 387 amino acid residues (with 100% similarity) of the type III export system. Using Blast search, the determined sequences of the five internal trypsin-digested peptides (Table 1) matched the amino acid sequence of flagellin from *Thermotoga maritima* MSB8 as shown in Fig. 2.

Table 1. Peptide sequences from *T. thermophilus* flagellin

Peptides	Peptidess	Amino acid sequence
1	(3-12)	**INHNIAAL NTLNR**
2	(39-51)	**AGDDAAGLAISEK**
3	(65-90)	**NSQDGI SLIQTAEGALTETHAILQR**
4	(233-270)	**AKLGAVQN RLEHTINNLS ASGENLTAAE SRIRDVDMAK**
5	(278-304)	**NNI LSQASQAMLA QANQQPQNVLQLLR**

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1   MRINHNISALNAWRNISQTQYSMSKTLERLSSGLRINHAGDDAAGLAISEKMRGQIKGLN 60
61  MAIKNAQDAISLIQTAEGALTEVHSILQRMRELAVQAASDTNTDVDREQIQKEIDQLREE 120
121 IDRIARTTEFNTKKLLDGKLESFRSQVDAKVVTGGNINVQLGSVSSAAVEGTYVIEVGQF 180
181 NGAETSELDVKITLFTAGGYSTTVVTITVGSATVGNINFTWDTDVLSINDFGGALPKNEV 240
241 VDSAVVRVEAIYTSASQLIFQIGANEGHNMVAGIDDMSAAALGLTTVSLDVTTQDAAERA 300
301 IMVVDAAIHRVSTARAALGAVQNRLEHTISNLGVAAENLTAAESRIRDADMAKEMMEFTK 360
361 QQILLQSSMAMLAQSNTLPQNVLQLMR 387
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Fig. 2: Peptides of *T. thermophilus* flagellin determined by MALDI-MS, matching with flagellin from *Thermotoga maritima* MSB8 (NCBI Accession number# NP_228567.1) as indicated in the boxes.

Discussion

Flagellin has been identified in various microorganisms as the product of the *hag* gene, which polymerizes to form the filaments of bacterial flagella and may have a role in translocation, secretion, or assembly of the flagellum. Carbon limitation in mineral medium has been reported to induce chemotaxis, which is indicated by the strong induction of *hag* and other flagellin specific genes involved in chemotaxis and motility in *B. subtilis* (Caramori T. and Galizzi A (1998). Flagellin and the *hag* mRNA levels were highly induced, especially from the middle to the end phase of growth in minimal medium (Mirel and Chamberlin, 1989). This increased expression denotes increases of carbon limitation in media containing, for instance, ammonia as a supplementary nitrogen source (Jürgen et al., 2005). The activation of the σ^B dependent gene expression during nutrient limited growth is triggered by a decrease of ATP. Thus, this response at high cell density conditions could be due to the decrease in the growth rate during this late stage of fermentation. In *B. subtilis,* amongst the σ^B operon gene mRNA that increased at the end of fermentation under high cell density conditions in medium containing ammonia as nitrogen source were genes involved in motility and chemotaxis, like the ones encoding for proteins involved in flagellin synthesis or processing and for GspA of the general stress regulon (Antelmann et al., 1995). In a wild-type strain of *B. subtilis hag* expression was low early in growth and increased exponentially during exponential growth in a complex medium, but it remained high and constant in a minimal medium (Mirel et al., 2000).

The PHB depolymerase/esterase exhibited maximal activity in the minimal medium used when the culture reached a high cell density. The co-purification of extracellular PHB depolymerase/esterase activity with flagellin is not surprising, since both proteins are secreted in the medium in a complexed form.

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