AMYLASE-NEGATIVE, ASPOROGENOUS MUTANT OF *BACILLUS SUBTILIS* USEFUL AS A HOST IN A HOST-VECTOR SYSTEM

FIELD OF THE INVENTION

This invention relates to a new amylase-negative mutant of *Bacillus subtilis* useful as a host into which vectors containing amylase-coding genes can be introduced using recombinant DNA methodology.

BACKGROUND OF THE INVENTION

Most genetic material in a bacterium exists as giant DNA molecules which are present as the chromosome of the cell. A certain amount of the genetic material may also be present in the form of smaller, closed circular DNA molecules known as plasmids. The portion of the DNA molecule related to a specific hereditary trait is called a gene.

By techniques referred to as genetic engineering, it is possible to transfer a gene, which codes for the production of a specific protein, from one microorganism to another. The microorganism which receives the new genetic material is referred to as the host. Various workers

have used these techniques to provide microorganisms which are superior producers of certain specific proteins such as enzymes.

It has been discovered that plasmids, which contain a series of genes linked together in the form of a circle, can be removed from the cells of one microorganism and inserted into the cells of another microorganism with comparative ease. Plasmids can also be used as vectors to carry new genetic material into a host organism. This is accomplished by first cutting the plasmid with an enzyme, known as a restriction endonuclease, that opens the circle of DNA. A fragment of foreign DNA, containing the desired gene, is inserted into the place where the DNA circle was cut. The circle is reformed by treatment with DNA ligase. The recombined plasmid, a new circular DNA molecule, contains the genes of the original plasmid plus the new gene from the piece of DNA which was inserted. This plasmid can be introduced into a host microorganism. The plasmid containing the new gene is then reproduced in the host microorganism and becomes part of its genetic material.

For a host microorganism to be suitable for use in genetic engineering, it must be capable of incorporating the new DNA. Furthermore, it must yield a viable microorganism which expresses the traits coded in the newly

inserted gene. For the microorganism to produce useful quantities of protein, the microorganism must also be one that can be grown on a commercial scale.

Experimenters using the new recombinant DNA technology have been concerned that the microorganisms with new genetic material might produce substances harmful to man, animals or plants. Because of this concern, the National Institute of Health (NIH) issued "Guidelines for Research Involving Recombinant DNA Molecules" in 1978. These guidelines provided for various levels of physical containment in laboratories where genetic engineering experiments are conducted. They also established levels of biological containment for microorganisms containing recombinant DNA.

Biological containment relates to the use of host cells and vectors which have limited ability to survive if they escape from the laboratories into the the natural environment. The NIH guidelines establish levels of biological containment for host-vector systems (hereafter designated HV) depending on the microorganisms and the genetic material used. HV1 is defined as a "host-vector system which provides a moderate level of containment". A high level of biological containment is required for a HV2 host-vector system.

The NIH has certified two mutants of B. subtilis for use as host components of HV1 systems: One of these, RUB331, is disclosed in U.S. Patent 4,302,544, the disclosure of which is incorporated herein by reference in its entirety. The second of these is BGSC No. IS53 which is described by Ellis, D. M. and Dean, D. H., Recombinant DNA Technical Bulletin, Vol. 4, No. 1, March, 1981. The NIH has approved one B. subtilis host component for a HV2 system. This strain, known as ASB298, is described by Burke, W. F. and Le, H. T., Recombinant DNA Technical Bulletin, Vol. 3, No. 1, December, 1980.

The present invention describes a new asporogenous mutant designated as B. subtilis EE101 (ATCC 39,096) useful as a host in a host-vector system. This host has the additional advantage of being an amylase-negative mutant. Such a mutant is particularly suitable for use as a host for recombinant plasmids which contain genes coding for the production of specific amylases such as thermostable alpha-amylases. These amylases are of commercial importance for use in processes to make starch hydrolyzates, glucose, and high fructose syrups. Since the mutant does not require an antibiotic for growth, it is a more practical host for enzyme production than the approved HV2 host, ASB298, which grows only in the presence of streptomycin.

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SUMMARY OF THE INVENTION

In accordance with this invention, there is provided a biologically pure culture of asporogenous B.subtilis EE101 (ATCC 39,096) suitable for use as a host component in a host-vector system characterized in that it contains no amylase-coding gene, has a frequency of reversion to spore formers of less than about 10^{-7} when grown under conditions of aeration and having the following genetic markers: thy A1, thy B1, pyr D1, spo 0AΔ677, lin-2, amy-3, and aro-10.

In addition, there is provided a biologically pure culture of amylase-negative B. subtilis EE1 (ATCC 39,093) useful as an intermediate for preparation of amylase-negative host components of host-vector systems characterized in that it contains the following genetic markers: lin-2, aro-10, and amy-3.

DETAILED DESCRIPTION OF THE INVENTION

The B. subtilis strains disclosed and claimed were prepared by incorporating the genetic material from other strains of B. subtilis. An amylase-negative strain of B. subtilis, ATCC 31,785(sample deposited on 5th January, 1981) containing the markers: aro-10 and amy-3, was transformed into a lincomycin-resistant strain by incorporation of the lin-2 marker from B.subtilis Strain 1A221 (ATCC 39,086) using a transformation reaction.

Strain A1221 was reported by Goldthwaite, Dubnau and Smith. Proc. Natl. Acad. Sci., U.S.A., 65, 96-103 (1970). It is available from the American Type Collection, Rockville, Maryland, as ATCC 39,086.

The resultant transformant, designated as EE1, contains the markers: lin-2, aro-10, and amy-3. It is available from the American Type Culture Collection as ATCC 39,093. This amylase-negative strain is useful as an intermediate for the preparation of amylase-negative host components of host-vector systems.

A portion of the DNA from B. subtilis Strain EE1 was then transferred into an asporogenous parent strain designated as DE101. Transfer was accomplished using a transduction process by means of the phage PBS1. Strain DE101 (ATCC 39,095) is disclosed in British Patent Application No. 8310741.

A stable mutant, EE101, possessing the markers: lin-2, aro-10, amy-3, thy A1, thy B1, pyr B1, and spo OAA677, resulted from the transduction.

The asporogenous, amylase-negative strain of the present invention, EE101, shows a frequency of reversion to spore formers of less than about 10^{-7}. It is able to grow under industrial conditions not requiring expensive growth requirements. It has a low survival rate under natural or escape conditions and a very low tendency to transmit plasmids to other organisms by natural genetic transfer. Although the organism shows a low degree of competence when subjected to classical transformation techniques, excellent transformation has been achieved using a protoplast transformation procedure. It functions well as a host for various plasmid vectors making it a useful strain to use as a host component of a B. subtilis host-vector system. Being amylase-negative, it is particularly suitable for use as a host for recombinant plasmids which contain genes coding for the production of amylases.

A detailed genetic map of the B. subtilis chromosome has been published by Henner and Hoch, Microbiological Reviews, 44, 57-82 (1980). Brief descriptions of the genetic markers mentioned in the disclosure of this invention follow:

(1) thy A1, thy B1: These mutations are in genes for two different thymidylate synthetases in B. subtilis. Both must be present to confer requirement for thymine and thymidine. This

requirement reduces the ability of the host to survive in nature. The thy A1 mutation also gives trimethoprim resistance, a useful marker for identifying a host.

(2) pyr D1: This mutation prevents formation of an enzyme essential to the production of pyrimidine by the microorganism. Since pyrimidines are essential precursors of DNA and RNA and are not commonly found in nature, starvation for pyrimidines will affect the growth of the host and the production of plasmid vehicles introduced into the host.

(3) spo OAΔ677: This is a deletion mutation causing a block in the earliest stages of sporulation. This mutation destroys the ability of the Bacillus to form spores, the form in which it normally survives in nature when subjected to heat, ultraviolet light, chemicals and desiccation.

(4) aro-10: This is a mutation which causes the mutants to require phenylalanine, tyrosine, and tryptophan. When deprived of these amino acids, a strain carrying this mutant ceases to grow.

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(5)amy-3 : This marker is associated with a deficiency in the structural gene for alpha-amylase production. It is characterized by a very low reversion frequency.

(6)lin-2: Strains containing this genetic marker will grow on plates containing minimal medium supplemented with appropriate amino acids and containing lincomycin at a concentration of 100 µg/ml. Strains which do not contain the marker will not grow on such plates.

The following example illustrates certain embodiments of the present invention. Unless otherwise stated, all proportions and percentages are provided on the basis of weight. Strains of microorganisms bearing ATCC numbers 39086, 39093, 39095 and 39096 were deposited at the American Type Culture Collection, Rockville, Maryland on 6th April,1982. All reagents bearing the Difco name are available from the Difco Laboratories, Detroit, Michigan.

EXAMPLE

Transformation of B. subtilis Strain ATCC 31,785 into a lincomycin-resistant strain was accomplished by incorporating DNA obtained from B. subtilis Strain 1A221 (ATCC 39,086) as follows.

Cells of B. subtilis ATCC 31,785 were grown overnight on agar plates containing Spizizen's minimal medium. Spizizen's minimal medium is a solution of (a) ammonium sulfate - 0.2%; (b) potassium phosphate (dibasic) - 1.4%; (c) potassium phosphate (monobasic) - 0.6%; (d) sodium citrate - 0.1%; and (e) magnesium sulfate - 0.02%; pH adjusted to 7.4. Cells from these plates were inoculated into 30 ml of a solution prepared by adding 0.1 ml 10% $MgSO_4$, 1.2 ml 50% glucose, 1.0 ml 10% Difco yeast extract and 1.0 ml 2% Difco casein hydrolyzate to 99 ml of Spizizen's minimal medium. The flask was incubated at 37°C and shaken at 200 rpm. The increase in optical density was monitored using a Klett spectrophotometer with a red filter. When cultures reached the transition between log and stationary growth (change in optical density of less than 5% in 15 minutes), they were diluted into 100 ml of the growth medium to which had been added 1.0 ml of 0.05 M $CaCl_2$ and 2.0 ml of 0.1 M $MgCl_2$. The mixture was incubated for an additional 90 minutes before donor DNA was added. ("Difco" is a Registered Trade Mark.)

Donor DNA was obtained from B. subtilis 1A221 (ATCC 39,086) by the following procedure.

Strain 1A221 was grown overnight at 37°C in a Heart Infusion Broth (BHI) medium available from Difco Laboratories, Detroit, Michigan. The mixture was diluted

with 500 ml of fresh BHI medium and grown at 37°C until the optical density of the mixture measured at 660 nanometers was 0.6. The cells were collected by centrifugation and resuspended in 10 ml of a solution containing 0.03 M tris(hydroxymethyl)aminomethane at pH 8.0, 0.005 M ethylenediamine tetraacetic acid and 0.05 M NaCl, and mixed with 4 mg of lysozyme. The cells were shaken gently (100 rpm) for 20 minutes at 37°C. Then 80 µl of pronase (10 mg/ml) was added and incubation was continued for another 60 minutes. The cells were then lysed by the addition of 1.0 ml of 20% sodium dodecyl sulfate solution. The lysate was extracted with phenol, phenol-chloroform, and chloroform, retaining the aqueous layer each time. DNA was precipitated by the addition of 3 volumes of cold 95% ethanol. The precipitated DNA was collected and resuspended in a solution containing 0.15 M NaCl and 0.015 sodium acetate at pH 7, and incubated at 37°C for 1 hour with a mixture of 1 mg pancreatic ribonuclease (type 1A from Bovine Pancreas, available from Sigma Chemical Company, St. Louis, Missouri) and 5 units of Ribonuclease T1 (Grade IV from Aspergillus oryzae, Sigma Chemical Company). The mixture was then re-extracted with phenol, phenol-chloroform, and chloroform and reprecipitated with ethanol. The DNA was redissolved in a solution containing 0.015 M sodium chloride and 0.0015 sodium acetate at pH 7, dialyzed against 3 changes of a large volume of the same solution during a 24-hour period before it was used for transformation.

Competent cells of B. subtilis ATCC 31,785, prepared as described above, were mixed with DNA at a concentration of 10 µg/ml of final mixture and the culture was shaken gently (100 rpm) for 60 minutes at 37°C. The culture was then diluted with 10 volumes of BHI and allowed to grow for an additional 3 hours at 37°C. The cells were collected by centrifugation, washed once with distilled water and spread on plates containing agar with Spizizen's minimal medium supplemented with all nutritional requirements plus lincomycin at a concentration of 100 µg/ml. A colony which grew was selected and designated as EE1. It had the markers: lin-2, aro-10, and amy-3. A culture of this strain is on deposit at the American Type Culture Collection as ATCC 39,093.

Strain DE101 (thy A1, thy B1, pyr D1, spo OAΔ677) was converted into the instant asporogenous strain, EE101, by transduction with phage PBS1 using DNA from the donor strain, EE1. Transduction was carried out by the method of Young and Wilson, Handbook of Genetics, Vol. 1, pp. 69-78, Plenum Press, New York, 1974, except that no chloramphenicol was added. Cells showing lincomycin resistance were selected by growing cultures on agar plates containing Spizizen's minimal medium supplemented with thymine (50 µg/ml), uracil (20 µg/ml) plus lincomycin (100 µg/ml) and the aromatic amino acids: phenylalanine, tyrosine, and tryptophan at a concentration of 20 µg/ml.

Three screening tests were applied to test for asporogenous mutants. First, the cells must remain unpigmented when grown on DSM plates (Sonenshein, et al, J. Bacteriology, 120, 253-265 (1974)). In contrast, spore-forming cells developed a dark reddish-brown color after 3 days' growth on these plates. DSM is Difco Sporulation Medium containing 0.8% nutrient broth, 0.025% $MgSO_4 \cdot 7H_2O$, 0.1% KCl, 1×10^{-6} M $FeSO_4$, 1×10^{-5} M $MnCl_2$ and 1×10^{-3} M $CaCl_2$. Secondly, if spores are present in the culture, they appear as phase-bright bodies in individual cells under a phase microscope. In contrast, asporogenous strains fail to develop the phase-bright stage. In the third test, the spores that are present in the culture, survive a heat treatment of 60°C for 20 minutes. In contrast, all cells in the culture of an asporogenous strain are susceptible to killing by heat in this test. For a strain to be classified as asporogenous, it must pass all three of the screening tests.

A biologically pure strain obtained by this process was designated as EE101. It has the genetic markers: lin-2, aro-10, amy-3, thy A1, thy B1, pyr D1, and spo OAΔ677. Strain EE101 is available from the American Type Culture Collection as ATCC 39,096.

EE101 requires mineral salts containing ammonium, potassium, phosphate and sodium ions for growth. It will utilize various carbon sources including glucose. Thymine or thymidine, pyrimidines and the aromatic amino acids: phenylalanine, tyrosine, and tryptophan, are required for growth. Corn steep liquor can be used as the source of these requirements but the addition of thymine to this liquor enhances growth of the organism.

Stability of the auxotrophic markers in Strain EE101 to reversion was determined by the following method. The strain was grown overnight at 37°C in Spizizen's minimal medium which was supplemented with 0.1% glucose, 10 µg/ml of each aromatic amino acid and uracil, and 50 µg/ml of thymine. Cultures were transferred to fresh media and allowed to grow for 6 hours with shaking at 37°C. The cells were then collected by centrifugation, washed twice in water, resuspended in water and titered on agar plates containing the minimal medium lacking only one of the requirements. Total viable cells were determined as colony forming units (CFU) on minimal plates containing all requirements. All determinations were done in triplicate. The data in Table I show the stability of the auxotrophic markers in the strain.

TABLE I

REVERSION FREQUENCIES OF MUTATIONS IN EE101

Mutation	CFU/ml (V)	Revertants/ml (R)	R/V
thy A1, *thy* B1	$1.9 \pm 0.26 \times 10^8$	0	$<10^{-8}$
pyr D1	$1.9 \pm 0.26 \times 10^8$	3.3 ± 0.6	1.7×10^{-8}
spo 0AΔ677	$1.9 \pm 0.26 \times 10^8$	0	$<10^{-8}$
aro-10	$1.9 \pm 0.26 \times 10^8$	6.7 ± 0.6	3.5×10^{-8}

Strain EE101 does not revert to spore formers even under conditions of adequate aeration which allow the cells the fullest possibility to sporulate. Cells were grown in DSM (Difco) medium supplemented with 50 µg/ml thymine at 37°C with shaking at 200 rpm. At 24-hour intervals, aliquots were removed and heated at 60°C for 20 minutes. The heated samples were then titered to determine the number of survivors. Comparisons were made with the number of CFU/ml developed when unheated samples were titered. The results shown in Table II indicate that no heat-resistant cells could be recovered from EE101 grown under these optimized sporulation conditions.

TABLE II

FORMATION OF HEAT-RESISTANT CELLS (SPORE FORMATION) OF EE101

Days	CFU/ml	Spores
1	4.7×10^8	0
2	1.1×10^8	0
3	7.0×10^7	0

Survival of EE101 in liquids was assessed as follows. Mid-log cultures were used to inoculate samples of BHI, Spizizen's minimal medium plus 0.1% glucose and sterile distilled water. One-ml samples were placed in capped 15-ml test tubes which were shaken in a 30°C air bath. At 24 hour intervals, 3 tubes from each group were removed and titered for survivors. The results expressed in CFU/ml are given in Table III.

TABLE III

SURVIVAL OF EE101 IN LIQUID MEDIA

Time (days)	BHI CFU/ml	Minimal Medium CFU/ml	H_2O CFU/ml
0	$1.01 \pm 0.15 \times 10^9$	$7.0 \pm 1 \times 10^7$	$3.93 \pm 0.74 \times 10^8$
1	$6.93 \pm 0.6 \times 10^8$	$1.39 \pm 0.3 \times 10^3$	$9.67 \pm 2.5 \times 10^3$
2	$4.03 \pm 0.3 \times 10^8$	$2.2 \pm 0.36 \times 10^2$	$4.61 \pm 0.2 \times 10^3$
3	$6.33 \pm 1.15 \times 10^7$	$6.1 \pm 1.1 \times 10^3$	$4.13 \pm 0.1 \times 10^3$
4	$8.4 \pm 0.53 \times 10^6$	$3.33 \pm 1.2 \times 10^1$	$3.4 \pm 1.2 \times 10^2$

To assess the survivability of EE101 in a simulated laboratory bench-top spill, 0.25-inch diameter sterile concentration discs were saturated with an aqueous suspension of washed cells which have been grown to mid-log phase in BHI plus thymine. Discs were stored at 37°C in sterile petri dishes. At 24-hour intervals, 3 discs were rehydrated in 10 ml Spizizen's minimal medium and the sample was titered for survivors. The results expressed in CFU/ml given in Table IV are the average of counts made on 3 separate discs.

TABLE IV

SURVIVAL OF EE101 ON "BENCH TOP"

Time (days)	CFU/ml	Survival (%)
0	$2.53 \pm 0.21 \times 10^{8}$	100
1	$1.67 \pm 0.58 \times 10^{1}$	6.6×10^{-6}
2	0	0
3	0	0

As shown in Table IV, EE101 survives for only a short time under these conditions of desiccation. This furnishes additional evidence of its suitability for use as a biological containment host for recombinant DNA.

The work described herein was all done in conformity with physical and biological containment requirements specified in the NIH guidelines.

CLAIMS

1. A biologically pure culture of asporogenous B. subtilis EE101 (ATCC 39,096) suitable for use as a host component in a host-vector system characterized in that it contains no amylase-coding gene, has a frequency of reversion to spore formers of less than about 10^{-7} when grown under conditions of aeration and having the following genetic markers: thy A1, thy B1, pyr D1, spo 0AΔ677, lin-2, amy-3, and aro-10.

2. A biologically pure culture of amylase-negative B. subtilis EE1 (ATCC 39,093) useful as an intermediate for preparation of amylase-negative host components of host-vector systems characterized in that it contains the following genetic markers: lin-2, aro-10, and amy-3.