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Transformation-Deficient Mutants of *Bacillus subtilis* Impaired in Competence-Specific Nuclease Activities

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A comparison of the nucleolytic activities in competent and physiologically low-competent wild-type cultures of *Bacillus subtilis* in DNA-containing sodium dodecyl sulfate-polyacrylamide gels revealed the existence of three competence-associated nuclease activities with apparent molecular weights of 13,000, 15,000, and 26,000. The three activities, which were dependent on manganese or magnesium ions, were specifically present in the competent fraction of a competent culture. The competence-associated nucleolytic activities of eight transformation-defective mutant strains were assayed, resulting in the following three classes of mutants: (i) four strains which, according to this assay, were not impaired in any of the nucleolytic activities mentioned above; (ii) one strain which was strongly impaired in the 13,000- and 26,000-molecular-weight activities, but showed a considerable level of the 15,000-molecular-weight activity; and (iii) three strains which were severely impaired in all three activities. The results indicated that the 26,000-molecular-weight activity was a dimer of the 13,000-molecular-weight activity and that this nuclease was involved in the entry of DNA.

Understanding of the mechanism of entry of transforming DNA into competent cells was greatly advanced through a series of studies by Lacks and co-workers (14, 15), who established that the major, membrane-located endonuclease is required for entry of DNA into competent *Streptococcus pneumoniae*. During entry the bound double-stranded DNA is converted to single strands, accompanied by partial acid solubilization of the DNA, both in *S. pneumoniae* (12, 13, 20) and in *Bacillus subtilis* (4, 5, 7, 9, 22). In both bacterial transformation systems entry of DNA can be inhibited by EDTA (19, 26). These similarities between the two systems of transformation suggest that a cell surface-located nuclease may also be involved in the entry of DNA in *B. subtilis*. To test this hypothesis, we examined the nuclease composition of physiologically low-competent and competent wild-type cultures, that of the competent and non-competent fractions of a competent wild-type culture, and that of a series of transformation-deficient strains which are impaired in the entry of DNA (2, 21). The results of this analysis showed that two nucleases are competence specific and that one is probably involved in the entry of DNA.

Some of these results were presented at the 5th European Meeting on Bacterial Transformation and Transfection, Florence, Italy, 1980.

MATERIALS AND METHODS

Bacterial strains. All strains used in this study were derivatives of *B. subtilis* 168 strain 8G-5 (1) and have been described previously (2, 21).

Media. Broth (pH 7.2) contained 1% tryptone (Difco Laboratories, Detroit, Mich.), 0.5% yeast extract (Difco) and 0.5% NaCl. Physiologically low-competent cells were prepared in MTY medium, consisting of Spizizen minimal salts (28) plus 0.5% glucose, 0.04% casein hydrolysate, growth factors at 28 μg/ml each (except for nicotinic acid and riboflavin, which were at 1.4 μg/ml), 10% (vol/vol) broth, and the vitamins biotin (6 ng/ml), aneurine hydrochloride (30 ng/ml), *p*-aminobenzoic acid (30 ng/ml) (Difco), calcium pantothenate (15 ng/ml) (Nutritional Biochemicals Corp., Cleveland, Ohio), pyridoxin hydrochloride (150 ng/ml), and cyanocobalamine (60 ng/ml) (Nutritional Biochemicals). Unless stated otherwise, all chemicals were obtained from British Drug House (BDH), Poole, England.

Isolation of DNA, preparation of competent and physiologically low-competent cultures, and transformation. The procedures for preparation of competent cells, isolation of DNA, and transformation were the same as described previously (21). Physiologically low-competent cultures were obtained by dilution of overnight cultures to an optical density at 450 nm of approximately 0.5 in MTY medium and growth for 5 h at 37°C with moderate aeration.

Preparation of spheroplast supernatant. Cells were harvested by centrifugation (10 min at 6,000 × *g* and 4°C) and washed once with 50 mM Tris-hydrochloride

(pH 7.4) containing 5 mM $MgCl_2$. Cells (2 to 4 g [wet weight]) were subsequently incubated in 50 ml of the same buffer containing 10 mM 2-mercaptoethanol, 20% sucrose, 0.7 mM α-toluenesulfonyl fluoride (E. Merck AG, Darmstadt, Germany), 2.5 mM *N*-ethylmaleimide (BDH), and 300 μg of lysozyme (Merck; 15,000 U/mg) per ml. Spheroplast formation was followed by phase-contrast microscopy. After 45 to 90 min of incubation at 37°C, spheroplasting was complete and the spheroplasts were removed by centrifugation (three times for 10 min each at 17,000 × *g* and 4°C). The supernatant was concentrated 5- to 10-fold by ultrafiltration at 0°C. The concentrate was dialyzed at 4°C against the buffer described above without $MgCl_2$ and lysozyme but containing 50% glycerol. Small portions were frozen at −80°C.

Preparation of membrane vesicles. The method used for the isolation of membrane vesicles was essentially that described by Konings et al. (11), with some modifications according to Konings (personal communication). Cells were harvested by centrifugation (10 min at 6,000 × *g*), and 4 g of cell paste was suspended in 100 ml of 50 mM potassium phosphate (pH 8.0) at 37°C. This suspension was mixed with 900 ml of the same buffer containing 250 mg of lysozyme, 15 mg of RNase (Miles Laboratories, Ltd., Slough, England), and 15 mg of DNase I (Miles) at 37°C. After addition of 10 mM $MgSO_4$ and incubation for 30 min at 37°C, 15 mM EDTA was added, resulting in almost complete clearing of the suspension. After 1 min, $MgSO_4$ was added (final concentration, 30 mM), and the suspension was centrifuged for 45 min at 25,000 × *g* and 4°C. The pellet was suspended in 80 ml of cold (4°C) 100 mM potassium phosphate (pH 6.6) by means of a Potter-Elvehjem type homogenizer and recentrifuged for 30 min at 48,000 × *g* and 4°C. The vesicles were suspended in 10 ml of 50 mM Tris-hydrochloride (pH 7.4) containing 0.1 mM EDTA, 0.5% (vol/vol) 2-mercaptoethanol, 0.7 mM α-toluenesulfonyl fluoride, 2.5 mM *N*-ethylmaleimide, and 50% glycerol at 0°C and were quickly frozen in liquid nitrogen in small portions.

Separation of competent and noncompetent cells. The competent and noncompetent fractions of a competent culture were separated according to the method of Joenje et al. (8) with some modifications. A competent culture (2.5 liters) in enriched minimal medium (21) was centrifuged at 1 h after dilution with starvation medium for 10 min at 6,000 × *g* at 20°C. The cell paste was suspended in 105 ml of the culture supernatant by means of a Potter-Elvehjem type homogenizer. The suspension was mixed with about 60 ml of 65% (wt/vol) Angiografin (Schering AG, Berlin Bergkamen, Germany), and the refractive index was adjusted to 1.3723 (at 20°C) with either supernatant or Angiografin. Cellulose nitrate tubes were filled with 27-ml portions of the mixture, and a 2.5-ml cushion, consisting of a mixture (1:1) of 65% Angiografin and supernatant, was injected underneath. Centrifugation was for 30 min at 68,000 × *g* at 20°C in a fixed-angle rotor (type 30; Beckman Instruments, Inc., Fullerton, Calif.). The rotor was allowed to slow down without braking. Cells were collected from the top layer (competent cells) and from the cushion (noncompetent cells) by puncturing the tube wall with a syringe. The cells were diluted 10-fold in starvation medium, washed, and subsequently spheroplasted as described above. Samples of each fraction were used to determine their transformabilities.

Electrophoresis in DNA-containing SDS-polyacrylamide gels. The buffer system of Laemmli (16) was used. The method described here is a modification of the procedure reported by Rosenthal and Lacks (23). The electrode buffer consisted of 25 mM Tris plus 0.192 M glycine (pH 8.3), 0.1% sodium dodecyl sulfate (SDS) (Bio-Rad Laboratories, Richmond, Calif.), and 2 mM EDTA. The stacking gel contained 5% acrylamide (Merck), 0.133% *N,N'*-methylenebisacrylamide (BDH), 0.125 M Tris-hydrochloride (pH 6.8), 0.1% SDS, 2 mM EDTA, 10 μg of highly polymerized calf thymus DNA (BDH) per ml, 0.1% ammonium persulfate (BDH), and 0.05% (vol/vol) *N,N,N',N'*-tetramethylethylenediamine (BDH). The separating gel contained 15% acrylamide, 0.4% *N,N'*-methylenebisacrylamide, 0.375 M Tris-hydrochloride (pH 8.8), 0.1% SDS, 2 mM EDTA, 10 μg of DNA per ml, 0.1% ammonium persulfate, and 0.05% *N,N,N',N'*-tetramethylethylenediamine. The sealing gel consisted of 10% Cyanogum 41 (BDH) in the separating-gel buffer without DNA. The sample-loading buffer contained 0.125 M Tris-hydrochloride (pH 6.8), 4% SDS, 2 mM EDTA, 10% (wt/vol) 2-mercaptoethanol, 20% glycerol, 0.8% bovine serum albumin (fraction V; Nutritional Biochemicals) (heated for 15 min at 98°C to destroy contaminating enzyme activities), 1.4 mM α-toluenesulfonyl fluoride, 5 mM *N*-ethylmaleimide, and bromophenol blue. Before application to the gel, the samples were diluted in the buffer in which they were obtained so that they contained equal amounts of protein according to the method of Lowry et al. (17). The samples were heated in the presence of SDS to dissociate possible multimers completely (16). Unheated samples were also examined to identify nuclease activity which might be heat sensitive. For this purpose the samples were mixed with an equal volume of the sample-loading buffer, and then one-half of this mixture was heated for 5 min at 98°C. After standing for 15 min at room temperature, both heated and unheated samples were applied to the gel and electrophoresed at room temperature. Electrophoresis was terminated as soon as the bromophenol blue front had migrated to the bottom of the separating gel.

The molecular weights (MWs) of the nucleases were determined by means of protein standards of known MW, which, after being heated in sample-loading buffer without albumin, were coelectrophoresed with the cell fractions examined. After electrophoresis the section of the gel containing the protein standards was cut from the gel and stained in 0.25% Coomassie brilliant blue (BDH)–50% methanol–10% acetic acid for 2 h at room temperature. The gel was destained by two washings (15 min each) in 50% methanol–10% acetic acid, followed by incubation overnight in 5% methanol–7.5% acetic acid. After the background was destained, the gels were placed on a light box and photographed. The standard proteins (Boehringer, Mannheim GmbH, West Germany) used were bovine serum albumin (MW, 68,000), ovalbumin (45,000), chymotrypsinogen A (25,000), trypsin inhibitor (21,500), and cytochrome *c* (12,500).

Electrophoresis under nondenaturing conditions. Electrophoresis of native cell fractions was performed

in horizontal polyacrylamide gels without SDS and DNA. The electrode buffer consisted of 50 mM Tris-citrate (pH 7.0). The separating gel contained 6% acrylamide, 0.16% *N,N'*-methylenebisacrylamide, 10 mM Tris-citrate (pH 7.0), 0.05% ammonium persulfate, and 0.14% *N,N,N',N'*,-tetramethylethylenediamine. After polymerization, the gel was washed in 1 liter of gel buffer for 30 min at room temperature. The samples were applied to the wells directly. Wells in the middle of the gel were used to allow the proteins to move in either the anodal or the cathodal direction. Bromophenol blue was used as a tracking dye. Electrophoresis was carried out with a current of 95 mA at 4°C and terminated as soon as the tracking dye had migrated to 0.5 cm from the end of the gel.

Visualization of nuclease activity. After electrophoresis in a DNA-containing SDS-polyacrylamide gel, the gel was rinsed in distilled water and washed in 1 liter of 40 mM Tris-hydrochloride (pH 7.6) containing 0.02% sodium azide by gentle shaking at room temperature for 1 h. After the buffer was renewed, the gel was shaken gently overnight at 37°C and subsequently incubated in fresh buffer containing 1 μg of ethidium bromide (Merck) per ml and one of the following cofactors: $MgCl_2$, $MnCl_2$, or $CaCl_2$ (2 mM, unless stated otherwise). When $MgCl_2$ was used, it was also present during the washing procedure. Incubation was continued at 37°C. At regular intervals the gels were placed on top of a Chromatovue transilluminator C60 (Ultra Violet Products, Inc., San Gabriel, Calif.) and photographed. During this incubation, the position of nucleolytic activity is detectable as a dark band in a fluorescent background (23).

After electrophoresis under nondenaturing conditions, the gel was rinsed in 1 liter of distilled water and then washed in 40 mM Tris-hydrochloride (pH 7.6) containing 10 mM $MgCl_2$ or $MnCl_2$ and 0.02% sodium azide to remove citrate and to achieve saturation with bivalent cations. The gel was subsequently washed in the same buffer without the bivalent cations and finally was washed once more in this buffer with the cofactors in the appropriate concentrations. All washings were in 1-liter volumes for 30 min at room temperature. The gels were then placed in a dry tray and overlayered with 1% agarose in 40 mM Tris-hydrochloride (pH 7.6) to which DNA (10 μg/ml), the appropriate cofactors, and 0.02% sodium azide had been added after the temperature had dropped to approximately 50°C. After the agar had solidified, a small volume of the final washing buffer was added to prevent desiccation. The gels were incubated overnight at 37°C to allow the nucleases to diffuse into the DNA-containing agarose overlay and to degrade the DNA. Incubation was continued at 37°C in the final washing buffer containing 1 μg of ethidium bromide per ml. At regular intervals the gels were photographed as described above.

Assay of DNase activity in native spheroplast supernatant. The DNase activity was determined by measuring the acid solubilization of double-stranded *B. subtilis* [^{3}H]DNA. Spheroplast supernatant was isolated as described above and, before concentration, dialyzed against 50 mM Tris-hydrochloride (pH 7.4)–2 mM EDTA–10 mM 2-mercaptoethanol–0.7 mM α-toluenesulfonyl fluoride–5% glycerol. Concentrated spheroplast supernatant was dialyzed against the same buffer containing 50% glycerol and stored frozen at −80°C. Before being added to the reaction mixture, the spheroplast supernatants were diluted in this buffer so that they contained equal amounts of protein according to the method of Lowry et al. (17). The reaction mixture (0.25 ml) contained 10 μl of spheroplast supernatant (containing 39 μg of spheroplast supernatant protein) and 0.75 μg of [^{3}H]DNA (215,000 cpm/μg of DNA) in a buffer consisting of 50 mM Tris-hydrochloride (pH 7.4), 5 mM $MnCl_2$, 0.5 mM EDTA, 10 mM 2-mercaptoethanol, and 400 μg of bovine serum albumin (heated for 15 min at 98°C) per ml. After 30 min at 37°C, the reaction was terminated by the addition of 0.25 ml of cold highly polymerized calf thymus DNA (1 mg/ml) and 0.5 ml of ice-cold 9% perchloric acid. After 30 min at 0°C, the mixture was centrifuged for 30 min at 10,000 × *g* at 4°C. Aliquots (0.3 ml) of the supernatant were mixed with 2.5 ml of Hydro Luma (Lumac Systems Inc., Titusville, Pa.) to determine acid-soluble radioactivity in a Nuclear-Chicago Mark II or a Packard 460 CD liquid scintillation counter.

RESULTS

Identification of competence-associated nucleolytic activities in entry-proficient wild-type cells. Joenje and Venema (9) previously suggested that a competence-associated exonuclease is present in the spheroplast supernatant of competent cells of *B. subtilis*. Therefore, the nucleolytic activities in the spheroplast supernatants of both competent and physiologically low-competent wild-type cultures were compared in DNA-containing SDS-polyacrylamide gels to identify those which are competence associated (Fig. 1). After incubation for 6 h in the presence of $MnCl_2$ as cofactor, two competence-associated nucleolytic activities were present in the unheated spheroplast supernatant of a competent culture (Fig. 1A, lane 4, bands I and III), whereas only one of these was active in the heated fraction (Fig. 1A, lane 3, band III). The MWs of the nucleases of bands I and III were estimated to be 26,000 and 13,000, respectively. Spheroplast supernatant of a physiologically low-competent culture did not show either of these activities after this period of incubation (Fig. 1A, lanes 1 and 2). The finding that the MW of the nuclease present in band III was one-half of that of the nuclease active in band I, together with the observation that heating of the spheroplast supernatant in the presence of SDS resulted in the disappearance of the activity of band I and in the concomitant increase of the activity of band III (most clearly seen in Fig. 1A and C, lanes 3 and 4), might suggest that the band I nuclease is a dimer that only dissociates completely into its monomers active in band III upon heating in the presence of SDS. After prolonged incubation (5 days) of the same gel, a third competence-associated activity was present (Fig. 1B, lane 3, band II) with an apparent MW of 15,000. Figure 1B also shows that after prolonged incubation in

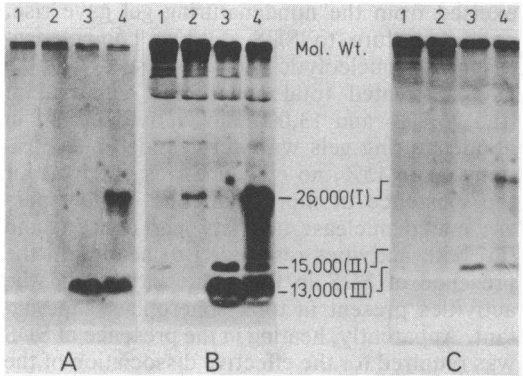

FIG. 1. Nuclease activities in spheroplast supernatants of competent and physiologically low-competent wild-type cultures after electrophoresis in a native DNA-containing SDS-polyacrylamide gel. (A) Incubation was for 6 h in buffer containing 2 mM $MnCl_2$. (B) Same gel as in (A) after 5 days of incubation. (C) Incubation was for 8 days in the presence of 2 mM $MgCl_2$. Lanes: 1, strain 8G-5, low competent, heated; 2, same as in lane 1, but not heated; 3, strain 8G-5, competent, heated; 4, same as in lane 3, but not heated. Portions of the same samples were used in both experiments shown in this figure. Portions containing 110 μg of spheroplast supernatant protein were applied to the wells. The MWs were derived from coelectrophoresis of protein standards of known MWs.

the presence of $MnCl_2$, the spheroplast supernatant of a physiologically low-competent culture also showed a weak activity at the positions of bands I (lane 2) and II (lane 1).

When a gel containing the same samples was incubated in the presence of $MgCl_2$ (Fig. 1C), the same competence-associated nucleolytic activities were present, but were considerably weaker than with $MnCl_2$. No additional competence-associated nucleolytic activities could be detected when $CaCl_2$ or ATP (50 μM in addition to 2 mM $MgCl_2$) were added as cofactors or when the gels were incubated at pH 9.0, which is near the optimal pH for certain *B. subtilis* nucleases (3, 6, 10, 27). In gels containing a lower acrylamide concentration (7.5%), which were used to separate more clearly the nucleolytic activities with high MWs, no significant competence-associated nuclease bands could be detected (data not shown).

Analysis of native competence-associated nuclease activities in spheroplast supernatant. One disadvantage of the nuclease assay described above is that it fails to detect the activities of nucleases requiring different subunits. Therefore, we also analyzed the nucleolytic activities of spheroplast supernatants not exposed to SDS, after electrophoresis in polyacrylamide gels not containing SDS. No DNA was included in these gels because positively charged nucleases might associate with the DNA in the gel, thus preventing their migration to their proper positions in the gel. Instead, after electrophoresis the gels were overlayered with 1% agarose containing the DNA and the cofactors, allowing nuclease activity to diffuse into the DNA-containing overlay. Figure 2 shows that the most striking difference in nucleolytic activity between the spheroplast supernatants of competent (lanes 4 and 5) and physiologically low-competent (lanes 1 and 2) wild-type cultures was the presence of a highly active manganese-dependent, competence-associated nuclease banding at R_f 0.55 in the anodal direction. When a gel containing the same samples was incubated in the presence of 10 mM $MgCl_2$, a much fainter competence-associated nuclease activity was visible at the same position in the gel. This activity was practically absent in 2 mM $MgCl_2$ and was completely absent when 2 mM $CaCl_2$ was used as cofactor. No additional competence-associated nuclease activities could be detected either when 0.2 mM ATP in addition to 2 or 10 mM $MgCl_2$ was added as the cofactor or when the gels were incubated at pH 9.0 in the presence of $MgCl_2$ with or without ATP (data not shown).

The competence-associated nuclease activity at R_f 0.55 was analyzed further. After excision

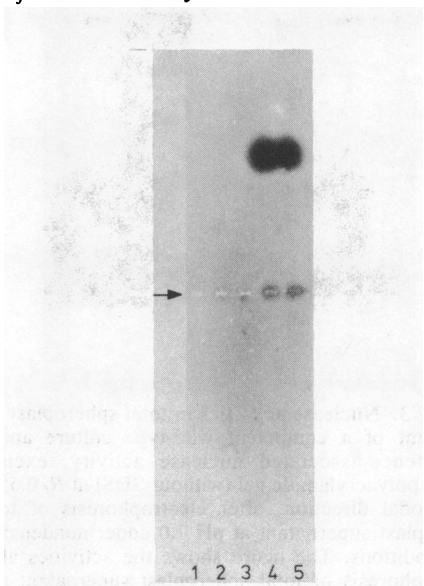

FIG. 2. Nuclease activities in spheroplast supernatants of competent and physiologically low-competent wild-type cultures after electrophoresis at pH 7.0 in a polyacrylamide gel without SDS. The samples were applied to the wells in the middle of the gel (arrow). The anode was at the upper side of the photograph. Incubation was for 24 h in the presence of 2 mM $MnCl_2$. Lanes: 1 and 2, strain 8G-5, low competent; 3, no sample; 4 and 5, strain 8G-5, competent. Portions containing 3 μg of spheroplast supernatant protein were applied to the wells.

from the nondenaturing gel, the gel segment containing the activity was cut and crushed through a 70-mesh stainless-steel screen (0.212 mm) (Retsch; GmbH & Co., Haan, Düsseldorf, Germany). The resulting slurry was shaken in buffer (50 mM Tris-hydrochloride [pH 7.4] containing 2 mM EDTA, 10 mM 2-mercaptoethanol, 0.7 mM α-toluenesulfonyl fluoride, 2.5 mM *N*-ethylmaleimide, and 10% glycerol) at 4°C to allow the enzyme to diffuse into the buffer. The extract was concentrated by ultrafiltration, incubated with SDS, and reelectrophoresed in DNA-containing SDS-polyacrylamide gels to determine whether this activity would yield nucleolytic activities as detected after electrophoresis of total spheroplast supernatant in these gels (Fig. 3). Upon incubation with SDS, the native nuclease gave rise to two nucleolytic activities with MWs of 26,000 and 13,000 (Fig. 3A, lane 4). When heated in the presence of SDS, the activity was predominantly recovered in a 13,000-MW polypeptide (Fig. 3A, lane 3). For the following reasons we presume that the 26,000-MW activity is a dimer consisting of two 13,000-MW subunits: (i) the native nuclease

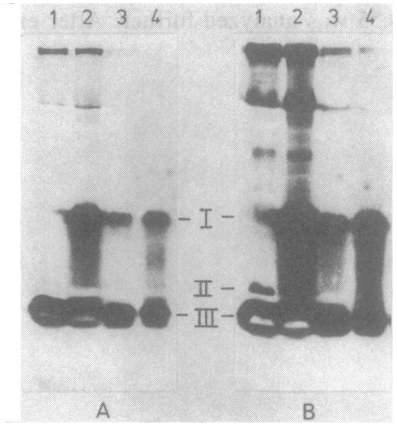

FIG. 3. Nuclease activities in total spheroplast supernatant of a competent wild-type culture and a competence-associated nuclease activity, excised from a polyacrylamide gel (without SDS) at R_f 0.55 in the anodal direction, after electrophoresis of total spheroplast supernatant at pH 7.0 under nondenaturing conditions. The figure shows the activities after electrophoresis of total spheroplast supernatant and the competence-associated nuclease activity in a native DNA-containing SDS-polyacrylamide gel. (A) Incubation was for 32 h in the presence of 2 mM $MnCl_2$. (B) Same gel as in (A) after 22 days of incubation. Lanes: 1 and 2, total spheroplast supernatant of strain 8G-5, competent; 3 and 4, excised competence-associated nuclease activity from the same spheroplast supernatant isolate. Lanes 1 and 3 contained the heated samples. Overexposure was necessary to show the presence of the 15,000-MW activity. See Fig. 1 for MWs of activities I, II, and III.

excised from the nondenaturing gel gave rise, upon exposure to SDS, to two competence-associated nucleolytic activities also detectable in SDS-treated total spheroplast supernatant (the 26,000- and 13,000-MW activities); (ii) in nondenaturing gels with acrylamide concentrations up to 15%, no evidence was obtained for the existence of more than one competence-associated nuclease activity near R_f 0.55; and (iii) both activities responded to heating in the presence of SDS in the same way as did the activities present in total spheroplast supernatant. Apparently, heating in the presence of SDS was required for the effective dissociation of the nuclease into its subunits. Figure 3B shows the same gel presented in Figure 3A but after prolonged incubation (22 days). It can be seen that the 15,000 MW-nucleolytic activity was absent in lane 3, whereas it was present under these conditions in total spheroplast supernatant of the same competent culture (lane 1). We believe, therefore, that this activity is not related to the native activity with R_f of 0.55. This assumption was supported by the results concerning the transformation-deficient strain 7G-321, which showed that the 15,000-MW activity was moderately impaired in this mutant, whereas the activities of MWs 26,000 and 13,000 were drastically reduced (see below).

Competence-associated nucleolytic activities in the competent and noncompetent fractions. To investigate whether the competence-associated nucleolytic activities are present in all cells of a competent culture or only in the competent fraction, a competent culture was separated into the competent and noncompetent fractions by means of Angiografin density gradient centrifugation. The nucleolytic activities in the spheroplast supernatants of both fractions were then compared by means of SDS-polyacrylamide gel-electrophoresis in DNA-containing gels (Fig. 4). The same competence-associated nucleolytic activities were present in spheroplast supernatant of the competent fraction (lanes 3 and 4) as in that of a nonfractionated competent culture (lanes 1 and 2). These activities were much weaker in the spheroplast supernatant of the noncompetent fraction (lanes 5 and 6). Probably the 26,000 (and 13,000)-MW activity present in the spheroplast supernatant of the noncompetent fraction is attributable to the low number of transformable cells present in this fraction: the difference in transformability between the competent and the noncompetent fractions amounted to a factor of 40 in the experiment shown here. These results indicate that both the nucleolytic activity of MW 26,000 (and 13,000) and that of MW 15,000 were competent cell specific.

Nucleolytic activities in the spheroplast supernatants of transformation-deficient strains. The

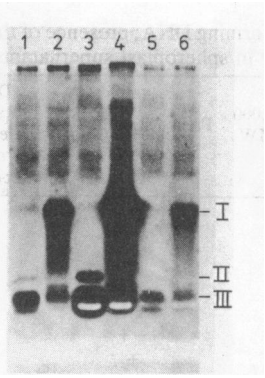

FIG. 4. Nuclease activities in spheroplast supernatants of a competent wild-type culture and of the competent and noncompetent fractions of a competent wild-type culture after electrophoresis in a native DNA-containing SDS-polyacrylamide gel. Incubation was for 19 days in the presence of 2 mM $MnCl_2$. Lanes: 1 and 2, strain 8G-5, competent; 3 and 4, competent fraction; 5 and 6, noncompetent fraction. Lanes 1, 3, and 5 contained the heated samples. Portions containing 6.5 μg of spheroplast supernatant protein were applied to the wells. See Fig. 1 for MWs of activities I, II, and III.

possible involvement of the competence-associated nucleolytic activities in the entry of transforming native DNA was investigated by analyzing the nuclease content of the spheroplast supernatants of a series of seven newly isolated transformation-deficient strains (21). The entry-deficient strain 7G-97, described by Buitenwerf and Venema (2), was also examined. Representative results are shown in Fig. 5. On the basis of comparison with the competent wild type (lanes 3 and 4), the strains could be divided in three classes: (i) strains that were not impaired in any of the nucleolytic activities that were detectable in this assay (strains 7G-97, 7G-315, 7G-320, and 7G-319; lanes 5 and 6); (ii) one strain (7G-321; lanes 7 and 8) showing a drastically reduced activity of the nuclease of MW 26,000 (and 13,000), but being only moderately impaired in the 15,000-MW nucleolytic activity; and (iii) strains that were strongly impaired in both activities (strains 7G-322, 7G-326, and 7G-325; lanes 9 and 10). In contrast to a physiologically low-competent wild-type culture (lanes 1 and 2), these strains retained some residual 26,000 (and 13,000)-MW activity. Because strain 7G-321 is strongly impaired in DNA entry, whereas its capacity to associate with DNA is only moderately impaired (Table 1), the strong reduction of the 26,000 (and 13,000)-MW activity suggests that it is involved in DNA entry. When the nucleolytic activities in spheroplast supernatants of the strains of classes ii and iii were analyzed in the presence of 2 mM $MgCl_2$, no competence-associated activities were detectable (data not shown).

The drastic impairment of the 15,000-MW nucleolytic activity in strains 7G-322, 7G-325, and 7G-326 in addition to their strongly reduced capacity to associate with DNA (Table 1), compared with strain 7G-321 which was only moderately impaired in this nucleolytic activity and still had a considerable DNA association capacity, suggests that this activity is involved in the association rather than in the entry of DNA.

DNase activity in native spheroplast supernatant. The capacity to degrade double-stranded *B. subtilis* DNA to acid-soluble material was determined in native spheroplast supernatant of the competent and physiologically low-competent wild type and of the eight transformation-deficient strains which had been exposed to the competence regimen (Table 1). On the basis of this analysis the strains could be divided in two classes: (i) strains which attained almost the wild-type level of DNase activity (strains 7G-97, 7G-315, 7G-319, and 7G-320) and (ii) strains which were strongly impaired in this activity (strains 7G-321, 7G-322, 7G-325, and 7G-326). The differences in DNase activity correlated fairly well with the level of the 26,000 (and 13,000)-MW nucleolytic activity assayed in DNA-containing SDS-polyacrylamide gels (Fig. 5).

Nucleolytic activities in membrane vesicles. In addition to the spheroplast supernatant, membrane vesicles were assayed for nucleolytic activities (Fig. 6). In the presence of $MnCl_2$, the competence-specific nuclease activities of MWs 26,000 and 15,000 appeared to be present in the

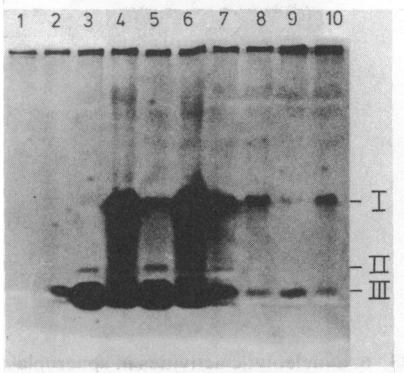

FIG. 5. Nuclease activities in spheroplast supernatants of wild-type and transformation-deficient strains after electrophoresis in a native DNA-containing SDS-polyacrylamide gel. Incubation was for 56 h in the presence of 2 mM $MnCl_2$. Lanes: 1 and 2, strain 8G-5, physiologically low competent; 3 and 4, strain 8G-5, competent; 5 and 6, strain 7G-319, competent; 7 and 8, strain 7G-321, competent; 9 and 10, strain 7G-325, competent. Lanes 1, 3, 5, 7, and 9 contained the heated samples. See Fig. 1 for MWs of activities I, II, and III.

TABLE 1. Total association, entry, and efficiency of entry of transforming DNA; presence of the competence-specific nucleolytic activities; and total DNase activity in spheroplast supernatants

B. subtilis strain	Total DNA association[a] (10^6 cpm/CFU)	Entry[b] (10^6 cpm/CFU)	Efficiency of entry[c] (%)	Presence of 26,000 (and 13,000)-MW nuclease[d]	Presence of 15,000-MW nuclease[d]	DNA-degrading activity[e] (cpm released per min/μg of spheroplast supernatant protein)
8G-5[f]				−	−	8.8
8G-5	241	53.6	100	++	++	79.6
7G-97[g]	183	2.3	5.6	++	++	57.5
7G-315	84.2	3.5	18.7	++	++	72.3
7G-319	152	0.6	1.8	++	++	81.8
7G-320	229	0.5	1.0	++	++	77.4
7G-321	35.9	0.3	3.8	±	+	10.0
7G-322	10.2	0.2	8.8	±	−	4.8
7G-325	5.7	0.2	15.8	±	−	13.3
7G-326	11.3	0.3	12.0	±	−	11.0

[a] Total amount of radioactivity, both sensitive and resistant to DNase I, associated with the cells of a competent culture after incubation for 30 min at 34°C with transforming [^{3}H]DNA (21). CFU, Colony-forming unit.

[b] Amount of DNase I-resistant radioactivity associated with the cells (21).

[c] Fraction of total cell-associated radioactivity rendered resistant to DNase I, expressed as percentage of the wild-type value (21).

[d] Presence of nucleolytic activity in DNA-containing SDS-polyacrylamide gels under the conditions described in the legend to Fig. 5. ++, Activity at wild-type level; +, activity slightly reduced; ±, activity strongly reduced; −, no activity detectable.

[e] Amount of [^{3}H]DNA rendered acid soluble during incubation for 30 min at 37°C with native spheroplast supernatant.

[f] Physiologically low competent.

[g] Total association, entry, and efficiency of entry of strain 7G-97 were determined as described previously (21).

membrane vesicles (lanes 5 and 6). The 13,000-MW activity in the heated sample (lane 5) was barely visible. However, the extent of activity in the 13,000-MW band appeared to be subject to considerable variation. No additional competence-associated nucleolytic activities were found when $MgCl_2$ was used as cofactor (data not shown).

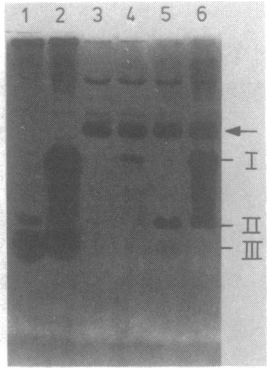

FIG. 6. Nucleolytic activities in spheroplast supernatant and membrane vesicles of wild-type cultures after electrophoresis in a native DNA-containing SDS-polyacrylamide gel. Incubation was for 4 days in the presence of 2 mM $MnCl_2$. Lanes: 1 and 2, spheroplast supernatant of strain 8G-5, competent; 3 and 4, membrane vesicles of strain 8G-5, physiologically low competent; 5 and 6, membrane vesicles of strain 8G-5, competent. Lanes 1, 3, and 5 contained the heated samples. The arrow indicates the position of DNase I which was added during the isolation of the membrane vesicles. See Fig. 1 for MWs of activities I, II, and III.

DISCUSSION

We have demonstrated the presence in spheroplast supernatant of three nucleolytic activities associated with the competent state. They were present in competent wild-type cultures, but only weakly so in physiologically low-competent wild-type cultures (Fig. 1). The results obtained after separation of a wild-type competent culture into the competent and noncompetent fractions strongly suggest that these nucleolytic activities are exclusively associated with competent cells (Fig. 4). Therefore, the residual activity in the low-competent cultures (Fig. 1B) is probably attributable to the low number of competent cells in such cultures. With respect to these competence-associated activities, two pertinent questions can be posed: first, what is their interrelationship, if any, and second, what is their significance in relation to the properties of the competent cell, viz., its capacities to bind and to take up transforming DNA? With respect to the first question, several observations suggest that the 26,000-MW nuclease is a dimer of the 13,000-MW nuclease and that the 15,000-

MW activity is independent of the former activities, at least at the protein level. Indications that the 26,000-MW activity is a dimer of the 13,000-MW activity are: (i) the disappearance of the 26,000-MW activity upon heating in the presence of SDS and the concomitant increase of the activity of 13,000 MW (Fig. 1A); (ii) the dissociation of the excised native competence-associated nuclease of R_f 0.55 upon exposure to SDS into a 26,000- and a 13,000-MW activity (Fig. 3); and (iii) the observation that in all mutants showing nuclease deficiencies, the 26,000- and 13,000-MW activities are both strongly impaired (Fig. 5).

The competence-associated nucleolytic activities of MWs 26,000 and 15,000 which were found in membrane vesicles obtained from competent wild-type cultures appeared to be the same as those in the spheroplast supernatant of such cultures because (i) the molecular weights did suggest so, (ii) these activities were also competence associated (Fig. 6), and (iii) they showed the same bivalent cation dependence, being much more active with manganese ions than with magnesium ions (data not shown). The observation that the 13,000-MW activity was only weakly active in membrane vesicles of competent wild-type cells, whereas the 26,000-MW nuclease showed a considerable activity (Fig. 6), seems to contradict the conclusion that these activities are interrelated. This may be explained by assuming that a membrane component inactivates the 13,000-MW polypeptide, either after disruption of the vesicles or during renaturation of the polypeptide if it had comigrated with the 13,000-MW activity.

Two arguments favor the idea that the 15,000-MW activity is not related to the 26,000-MW activity at the protein level: (i) the former activity was absent from the SDS-polyacrylamide nuclease pattern obtained from the excised native nuclease of R_f 0.55 (Fig. 3B) and (ii) it was still present, although slightly less active than in the wild type, in strain 7G-321, which is drastically impaired in the 26,000 (and 13,000)-MW activity (Fig. 5).

A comparison of the nuclease deficiencies of the mutant strains with their DNA-binding and DNA-entry capacities should provide information regarding the functions of the two nucleases in the transformation process. In the strains 7G-322, 7G-325, and 7G-326, both total association (binding plus entry) and entry are inhibited to a considerable extent (Table 1). In these mutants both the 26,000- and the 15,000-MW nucleases are strongly impaired, so it cannot be decided which one is specifically involved in the entry of transforming DNA. However, the properties of strain 7G-321 seem to make this decision feasible: it still possesses a fair binding capacity, is not much impaired in the 15,000-MW nuclease activity but is strongly inhibited in DNA entry (Table 1), and has retained very little 26,000-MW nuclease activity (Fig. 5). These properties suggest that the 26,000-MW activity is involved in the entry of DNA and, in addition, that the 15,000-MW activity is engaged in the binding of DNA. The latter suggestion is supported by the properties of strains 7G-322, 7G-325, and 7G-326, which are strongly impaired in the 15,000-MW nuclease activity and show a drastically reduced capacity to associate with DNA.

During transformation in *B. subtilis*, the cell-bound native donor DNA is converted into double-stranded fragments, having a MW of 9×10^6 or more, which are still sensitive to removal by exogeneously added DNase I (7). Two endonucleases have been isolated from *B. subtilis* that have been implicated in the formation of these double-stranded fragments. Scher and Dubnau (24, 25) have described an endonuclease which is released upon spheroplasting and generates double-stranded fragments of MW of 10^6 or more. However, it is unlikely that this nuclease is related to the 15,000-MW activity, because it is active not only with manganese but also with calcium ions as cofactor, whereas magnesium has no stimulating effect. In addition, this nuclease was isolated from physiologically low-competent cultures. McCarthy and Nester (18) and Burke and Spizizen (3) have described a heat-stimulated, magnesium-dependent endonuclease which was isolated from the total cell lysate of competent cultures. This nuclease also does not seem to be related to the 15,000-MW activity, because the endonuclease was much more active with magnesium ions than with manganese ions.

The major difference between the competence-specific nucleolytic activities in DNA-containing SDS-polyacrylamide gels of strain 7G-321 compared with those of strains 7G-322, 7G-325, and 7G-326 (Fig. 5) was the presence of the 15,000-MW nucleolytic activity in strain 7G-321. Therefore, the results obtained in the assay of DNase activity in native spheroplast supernatant (Table 1) suggest that this nuclease does not noticeably contribute to the production of acid-soluble material from double-stranded DNA. DNase activity assayed in this way seems to reflect predominantly the activity of the 26,000 (and 13,000)-MW nuclease.

It is unlikely that the 26,000-MW activity is the sole component effecting the entry of transforming DNA. This can be concluded from the properties of strains 7G-319 and 7G-320, which are not at all or only slightly impaired in DNA binding, but are strongly impaired in DNA entry (Table 1). Nevertheless, these mutants possessed a fully active 26,000-MW nuclease (Fig.

5). Apparently these strains are impaired in a component, probably not a nuclease, which is required for DNA entry. Because the breakdown of donor DNA to acid-soluble products is strongly reduced in these strains (21), it is conceivable that this component translocates the bound DNA to the entry-involved nuclease so that the entry process can be initiated.

Although the 26,000- and the 15,000-MW nucleases did not seem to be related at the protein level, the fact that in three of four of the strains showing nuclease deficiencies both activities were strongly impaired, notwithstanding previous purification of the mutations by transformation (21), indicates the existence of a relationship between the two activities at the genetic level. Although the nature of this relationship remains to be established, it is conceivable that the two proteins are specified by the same transcriptional unit and that the mutations in the three strains strongly impaired in the activity of the two proteins are either located in the regulatory part of the operon (its repressor locus) or are polar mutations in the structural gene of one of the proteins.

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