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Alternansucrase mutants of *Leuconostoc mesenteroides* strain NRRL B-21138

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Alternan is a unique α-D-glucan of potential commercial interest, produced by rare strains of *Leuconostoc mesenteroides.* Natural isolates that produce alternan, such as NRRL B-1355, also produce dextran as a troublesome contaminant. We previously isolated mutants of strain NRRL B-1355 that are deficient in dextran production, including the highly stable strain NRRL B-21138. In the current work, we mutagenized strain NRRL B-21138 and screened survivors for further alterations in production of alternansucrase, the enzyme that catalyzes the synthesis of alternan from sucrose. Second generation mutants included highly stable strain NRRL B-21297, which produced four-fold elevated levels of alternansucrase without an increase in the proportion of dextransucrase activity. Such alternansucrase overproducing strains will facilitate studies of this enzyme, and may become valuable for the enzymatic production of alternan. Another highly stable mutant strain, NRRL B-21414, grew slowly on sucrose with negligible production of glucan or extracellular glucansucrase activity. This strain may prove useful as an expression host for glucansucrase genes.

Keywords: alternan; alternansucrase; dextran; dextransucrase; glucan; glucosyltransferase

Introduction

Although many strains of *Leuconostoc mesenteroides* produce dextrans, only three naturally occurring strains out of hundreds examined produce alternan [4,6,13]. Dextrans from *L. mesenteroides* are α-D-glucans with backbone structures in which α-(1 → 6) linkages predominate [3]. Alternan is also an α-D-glucan, distinguished from dextran by a backbone structure of regular, alternating α-(1 → 6), α-(1 → 3) linkages [4,11,12]. This unique structure is thought to be responsible for alternan's distinctive physical properties of high solubility and low viscosity. Alternan and its derivatives have functional properties that resemble commercially valuable glucans such as gum arabic, maltodextrins, and Polydextrose™ [2]. Alternan thus has potential uses as a low-viscosity bulking agent or extender in foods and cosmetics.

However, all known naturally occurring strains of *L. mesenteroides* that produce alternan also produce dextran in significant amounts, and the separation of dextran from alternan is a relatively complicated and expensive process [2,4]. Alternan-producing strains secrete two distinct glucosyltransferases when grown on sucrose-containing media [4]. Alternansucrase catalyzes the transfer of D-glucopyranosyl units from sucrose to alternan. In the reaction scheme, the fructosyl moiety of the sucrose molecule is released. No costly organic cofactors are required, and the reaction is entirely extracellular. Dextransucrase promotes an analogous reaction, producing dextran and fructose from sucrose.

In fermentative production of alternan and dextran, fructose is taken up and consumed by cells of *L. mesenteroides*. Since fructose is commercially valuable, cell-free enzymatic synthesis of alternan may be an attractive alternative. Enzymatic production may offer other advantages as well, such as improved control of product quality. Alternan production has been demonstrated using immobilized alternansucrase [3]. However, available methods for purification of alternansucrase are complex and costly, and suffer from poor yields [4,10]. Furthermore, natural isolates produce only low levels of alternansucrase. Consequently, a major goal of our research has been the development of improved microbial sources of alternansucrase.

We recently isolated *L. mesenteroides* strain NRRL B-21138 as a highly stable derivative of alternan-producing strain NRRL B-1355 [9]. Strain NRRL B-21138 secretes alternansucrase with little or no contaminating dextransucrase. As a result, this mutant ferments sucrose to alternan with little detectable dextran. However, strain NRRL B-21138 produces alternansucrase at levels approximately equal to its parental strain. In terms of further strain improvements, we were thus interested in improving alternansucrase yields. We were also interested in obtaining mutants that produced neither dextransucrase nor alternansucrase, as potential hosts for cloned glucansucrase genes. In the current work, we mutagenized *L. mesenteroides* NRRL B-21138 and screened survivors for further alterations in glucan or alternansucrase production.

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Materials and methods

Culture maintenance, growth and mutagenesis

L. mesenteroides strain NRRL B-1355 was obtained from the ARS Culture Collection, Peoria, IL, USA. Strain NRRL B-21138 was derived from strain NRRL B-1355 as recently described [9]. *L. mesenteroides* strains were routinely maintained on medium containing 2.0% sucrose, 0.15% polypeptone, 0.15% beef extract, 0.15% yeast extract, 0.1% Tween 80, 0.2% ammonium citrate, 0.5% sodium acetate, 0.01% $MgSO_4 \cdot 7H_2O$, 0.005% $MnSO_4 \cdot H_2O$ and 0.2% K_2PO_4, pH 6.5. Solid medium contained 1.5% agar. Liquid cultures were grown at 28°C with shaking at 100 rpm. For mutagenesis, cells were harvested from midlog phase cultures (OD_{600} of 0.2–0.7) and resuspended in 10 mM $MgSO_4$ before exposure to approximately 330 μW cm^{-2} ultraviolet radiation for 10–20 s, resulting in more than 90% mortality. Survivors were diluted to produce isolated colonies on solid medium containing sucrose. Plates were incubated at 28°C in the dark for 1–2 days.

Mutant screening and characterization

Mutagenized survivor colonies that were atypical in size or mucoid appearance on sucrose-containing solid medium were chosen as putative alternansucrase mutants. These isolates were grown in liquid culture and total extracellular sucrase activity and glucan were measured. Derivatives exhibiting a non-parental phenotype were sequentially passed in liquid culture for more than 60 generation cycles and reassayed. Strains that retained a mutant phenotype after this extended period were considered genetically stable. Stable strains were assayed for glucansucrase activities, and alternansucrase proteins were characterized by gel electrophoresis.

Enzyme and glucan analyses

Liquid culture growth was monitored by optical density at 600 nm. Total extracellular glucan was quantified as the dry weight of ethanol-precipitated material from cell-free culture supernatants. Alternan and dextran were calculated as previously described, based on the preferential digestion of dextran by dextranase [4]. Total extracellular sucrase activity was estimated by the production of reducing sugar from sucrose [9]. Glucansucrase activity was measured as previously described [4], based on the incorporation of radiolabel from [U-^{14}C] sucrose into methanol-precipitable glucan. Alternansucrase activity was calculated based on the percentage of total precipitable radioactivity after exhaustive digestion by dextranase [4]. Reported values are means from replicate culture preparations, exhibiting standard deviations of no more than 12% of the mean.

Characterization of alternansucrase proteins

Alternanase is a recently discovered glucanase that specifically attacks alternan [1]. In order to dissociate alternan/alternansucrase complexes, 25-ml aliquots of cell-free culture supernatants were mixed with 5 ml partially purified alternanase preparation and incubated at room temperature for 60 h while dialyzing the mixture against 20 mM NaMOPS, pH 7.0, containing 1 mM $CaCl_2$ and 0.02% sodium azide. Dialyzed samples were concentrated approximately five-fold by ultrafiltration (Amicon, Beverly, MA, USA), mixed with sample buffer and heated for 10 min at 70°C just prior to SDS-PAGE on 4–12% gradient gels at pH 7.0 (Novex, San Diego, CA, USA). Replica gels were either silver-stained to reveal total protein patterns [16] or developed as glucansucrase activity gels. Activity gels were incubated overnight at 35°C in a substrate buffer containing 5.0% sucrose, 0.5% NP-40, 1.0 mM $CaCl_2$ in 50 mM NaMES at pH 5.2, with 0.02% sodium azide as a preservative. Under these conditions, glucansucrases renatured and catalyzed the formation of glucans in situ. Nascent glucan bands were precipitated in 50% methanol–10% acetic acid and stained using the periodic acid-Schiff's reagent [8].

Results and discussion

Strains used in this study

Strain NRRL B-1355 is a natural isolate of *L. mesenteroides* that produces both alternan and dextran in approximately equal amounts [4]. Strain NRRL B-21138 is a derivative of NRRL B-1355 that produces alternansucrase with little contaminating dextransucrase [9]. Strain NRRL B-21138 may be similar to other dextransucrase mutants described [14], but has been further characterized as highly stable [9]. In the current study, strain NRRL B-21138 was mutagenized and screened for further alterations in alternansucrase production. Strains NRRL B-21297 and NRRL B-21414 were independent derivatives of strain NRRL B-21138 that exhibited atypical colonial morphologies.

Colonial morphologies of alternansucrase mutants

Mutagenized survivor colonies were initially screened for atypical colonial morphologies that might suggest changes in glucan production. Colonies of strain NRRL B-21138 (Figure 1b) were extremely mucoid, and even larger and more spreading than colonies of natural isolate NRRL B-1355 (Figure 1a). This difference may reflect the fact that strain NRRL B-21138 primarily produced alternan, while strain NRRL B-1355 produced a mixture of alternan and dextran; alternan is less viscous and has greater water solubility than dextran [2]. Strain NRRL B-21297 (Figure 1c) produced colonies that were smaller and less mucoid than those of strain NRRL B-21138. Colonies of NRRL B-21414 (Figure 1d) arose only slowly on solid medium containing sucrose, and were essentially non-mucoid. Based on these colonial morphologies, strains NRRL B-21297 and NRRL B-21414 were initially assumed to be similar mutants that produced reduced levels of alternansucrase.

Growth and production of glucans and glucansucrases

Strains NRRL B-1355, NRRL B-21138, NRRL B-21297 and NRRL B-21414 were cultured in liquid medium containing sucrose. Strains NRRL B-1355 and NRRL B-21138 exhibited similar cellular morphologies. Typical of *L. mesenteroides*, cells were lenticular and most often in pairs, although single cells and short chains could be found. Strain NRRL B-21297 exhibited a strong bias toward

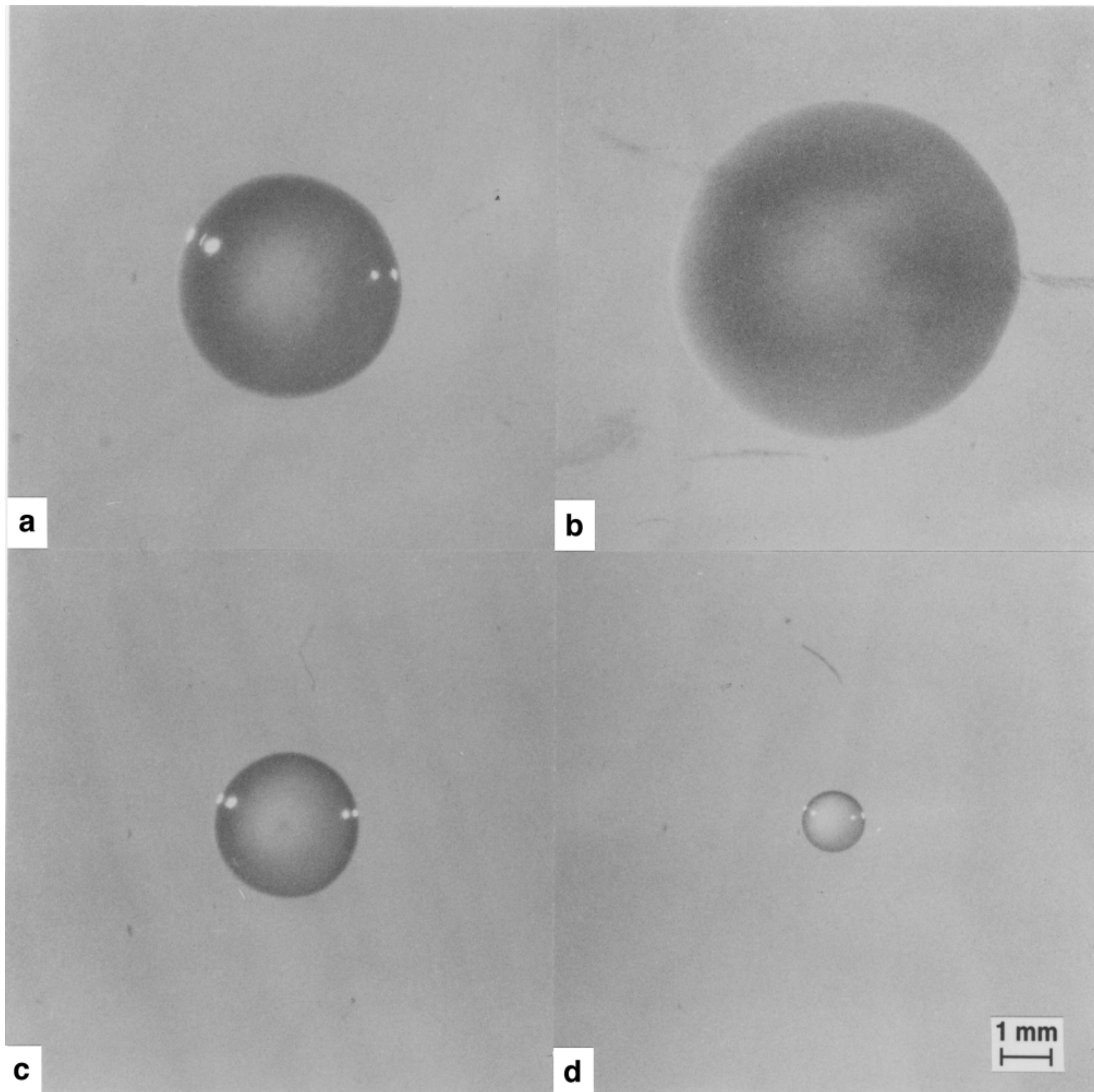

Figure 1 Colonial morphology of *L. mesenteroides* strains on solid medium containing sucrose. Panels: (a) strain NRRL B-1355; (b) strain NRRL B-21138; (c) strain NRRL B-21297; (d) strain NRRL B-21414.

growth in chains. Cells of strain NRRL B-21414 occurred primarily in clumps.

As shown in Figure 2, all strains with the exception of NRRL B-21414 grew after only a brief lag phase, and reached a stationary phase within 24 h. These results suggest that differences in glucansucrases among these strains did not limit growth. Strain NRRL B-21414 exhibited an extended lag period, and grew more slowly to a final growth yield similar to those of other strains.

Parental strain NRRL B-1355 produced approximately equal levels of alternansucrase and dextransucrase in liquid medium containing sucrose, and corresponding quantities of alternan and dextran (Figures 3 and 4), consistent with previous descriptions of this strain [4,6]. Strain NRRL B-21138 was isolated as a mutant derivative of strain NRRL B-1355 that produced alternansucrase with little dextransucrase [9]. Glucansucrase from strain NRRL B-21138 included alternansucrase at levels similar to NRRL B-1355, and only low levels of dextransucrase (Figure 3). Total glucan amounts produced by strains NRRL B-1355 and NRRL B-21138 were similar (approximately 25% of initial substrate), although glucan from strain NRRL B-21138 was predominantly composed of alternan (Figure 4).

Contrary to initial assumptions based on colonial morphology, strain NRRL B-21297 produced elevated levels of alternansucrase in liquid culture containing sucrose (Figure 3). Maltose-grown cultures of strain NRRL B-21297 produced negligible levels of glucansucrase (data not

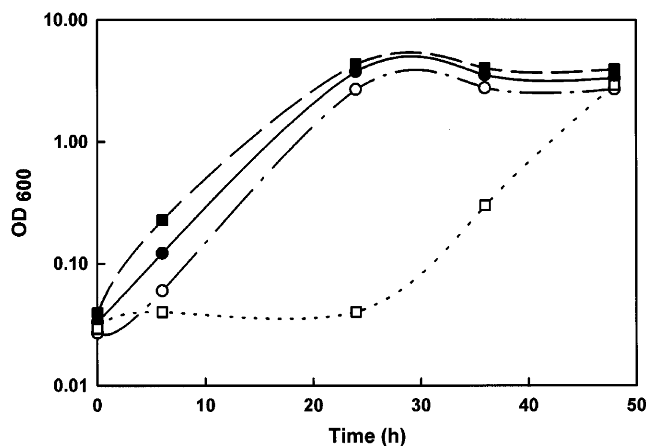

Figure 2 Growth of *L. mesenteroides* strains in liquid medium containing sucrose. Symbols: ●, strain NRRL B-1355; ■, strain NRRL B-21138; ○, strain NRRL B-21297; □, strain NRRL B-21414.

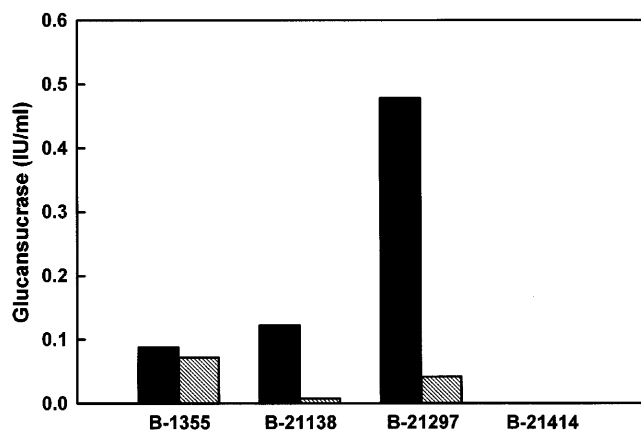

Figure 3 Glucansucrase production by 2-day cultures of *L. mesenteroides* on liquid medium containing sucrose. Solid bars: alternansucrase activity. Hatched bars: dextransucrase activity.

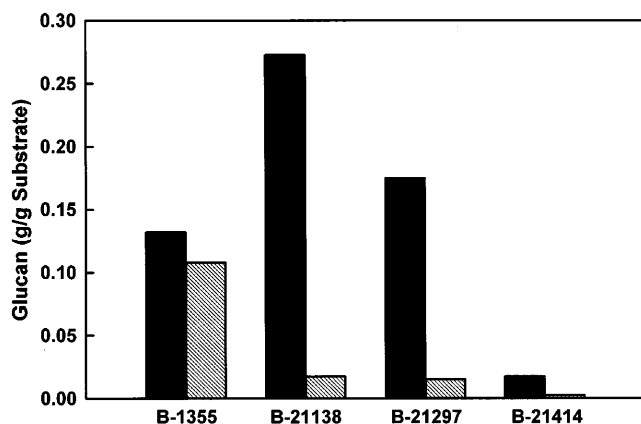

Figure 4 Glucan production by 2-day cultures of *L. mesenteroides* on liquid medium containing sucrose. Solid bars: alternan. Hatched bars: dextran.

shown). Strain NRRL B-21297 is thus not similar to constitutive alternansucrase mutants recently described [7]. Surprisingly, strain NRRL B-21297 produced alternan by fermentation in lower yields than did strain NRRL B-21138 (Figure 4). Since final glucan yields are limited by available substrate carbon rather than enzyme levels, this result suggests that strain NRRL B-21297 consumed more sucrose for growth and other metabolic activities, possibly including increased enzyme production, than did strain NRRL B-21138.

It is unclear how or whether the overproduction of alternansucrase is related to the colonial morphology of strain NRRL B-21297; non-mucoid mutants of *Streptococcus gordonii* that overproduced glucosyltransferase were reported by Haisman and Jenkinson [5]. It is conceivable that the atypical cellular morphology of strain NRRL B-21297 is related to its colonial morphology. It may also be that the reduced production of alternan contributes to the appearance of smaller, less mucoid colonies.

From an applied standpoint, strain NRRL B-21138 would thus be superior for production of alternan by direct fermentation of sucrose. On the other hand, strain NRRL B-21297 would be the strain of choice for production of alternansucrase, particularly for the subsequent enzymatic production of alternan. The high genetic stability of strains NRRL B-21138 and NRRL B-21297 would be important in commercial applications.

Strain NRRL B-21414 produced only very low levels of extracellular glucansucrase and glucan (Figures 3 and 4). The extended lag phase of this strain on sucrose (Figure 2) may reflect an induction of intracellular sucrases, such as sucrose phosphorylase [15]. Strain NRRL B-21414 may thus be useful as a host for the expression of isolated glucansucrase genes.

Alternansucrase proteins

Spent medium from cultures grown in sucrose were treated as described in Materials and Methods and analyzed by neutral pH SDS-PAGE. Silver-stained gel replicates showed that the secreted protein patterns among the strains were similar (Figure 5a). Levels of alternansucrase proteins were low relative to total secreted proteins, and individual alternansucrase proteins were difficult to discern. However, differences among strains could easily be seen by comparing the levels of glucan produced in activity replicate gels after incubation in sucrose (Figure 5b). Wild-type strain NRRL B-1355 produced significant amounts of glucan at two band positions corresponding to proteins of molecular weights 145 and 135 kDa (Figure 5, lane B1). Both glucans were resistant to dextranase digestion, and thus presumably corresponded to active alternansucrase species. Under experimental conditions used, active dextransucrase proteins were not detected. Dextransucrase is less stable than alternansucrase [4], and may be present on gels in an inactive form. Alternatively, dextransucrase, perhaps complexed with glucan, might be unable to enter the polyacrylamide.

Dextransucrase mutant strain NRRL B-21138 showed a pattern similar to that of parental strain NRRL B-1355 (Figure 5 lane B2). Strain NRRL B-21414 produced only a trace of stained glucan, consistent with the lack of glucansucrase activity seen in this strain (Figure 5 lane B3). Alternansucrase overproduction mutant strain NRRL B-21297 clearly produced elevated levels of glucan at both band positions (Figure 5 lane B4). In subsequent experiments, we

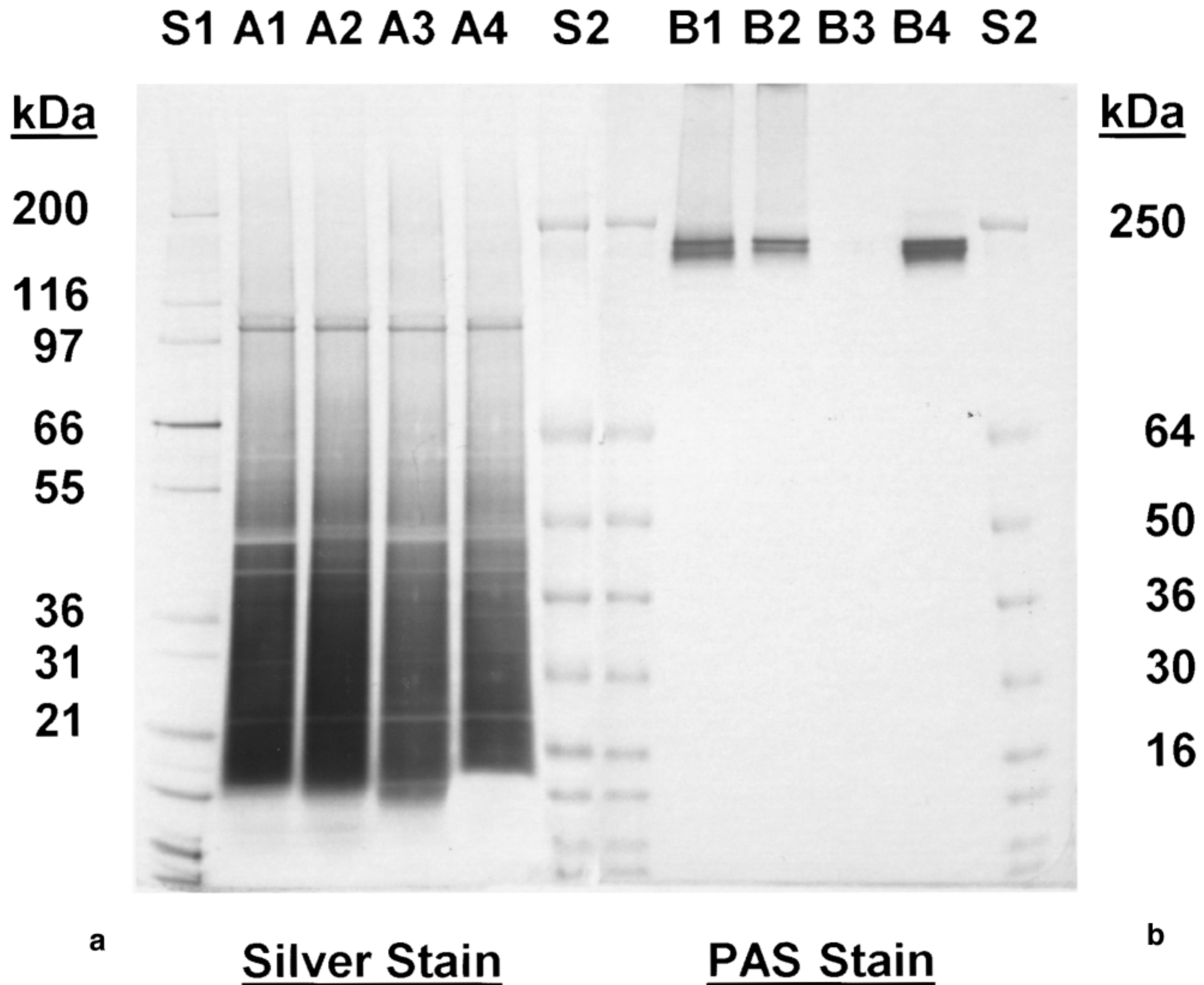

Figure 5 Neutral pH SDS-PAGE (4–12% gradient gel) of *L. mesenteroides* culture supernatants. The left half of gel (a) was silver-stained after electrophoresis to detect total proteins. The right half of gel (b) was incubated in a sucrose solution for 18 h prior to staining it with periodic acid/Schiff reagent, to detect glucansucrase activity. Lanes: A1/B1, strain NRRL B-1355 (wild-type); A2/B2, strain NRRL B-21138; A3/B3, strain NRRL B-21214; A4/B4, strain NRRL B-21297; S1, Novex Mark 12 molecular weight standards; S2, Novex See-Blue prestrained molecular weight standards. Molecular weights (kDa) are indicated.

have further separated the 145 and 135-kDa alternansucrase proteins from strain NRRL B-21297 and demonstrated that they are distinct, although possibly related, species. Interestingly, strain NRRL B-21297 also produced a minor amount of glucan at positions corresponding to two proteins of approximately 250 kDa. We are currently examining the apparent heterogeneity among glucansucrases from these strains.

Conclusions

Alternan is a novel biopolymer with physical properties of potential commercial interest. Practical applications using alternan have been stymied by the difficulties involved in separating alternan from co-produced dextrans. Similarly, alternansucrase has been difficult to separate from dextransucrase produced by naturally occurring strains of *L. mesenteroides*. The availability of alternansucrase mutants in a genetic background free of dextransucrase will facilitate further studies of this enzyme, and expedite feasibility studies of commercial applications for alternan.

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