Biofilms of clinical strains of *Staphylococcus* that do not contain polysaccharide intercellular adhesin

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Abstract

Staphylococcus aureus and coagulase-negative staphylococci, primarily *Staphylococcus epidermidis*, are recognized as a major cause of nosocomial infections associated with the use of implanted medical devices. The capacity of *S. epidermidis* to form biofilms, allowing it to evade host immune defence mechanisms and antibiotic therapy, is considered to be crucial in colonizing the surfaces of medical implants and dissemination of infection. It has previously been demonstrated that the biofilm of a model strain *S. epidermidis* RP62A comprises two carbohydrate-containing moieties, a polysaccharide having a structure of a linear poly-*N*-acetyl-(1 → 6)-β-D-glucosamine and teichoic acid. In the present paper we show that, unlike this model strain, certain clinical isolates of coagulase-negative staphylococci produce biofilms that do not contain detectable amounts of poly-*N*-acetyl-(1 → 6)-β-D-glucosamine. In contrast to that of *S. epidermidis* RP62A, these biofilms are not detached with metaperiodate, while proteinase K causes their partial dispersal.

Introduction

Staphylococcus aureus and coagulase-negative staphylococci (CoNS) including *Staphylococcus epidermidis* as a leading species have emerged as the most frequently isolated pathogens in nosocomial sepsis and currently cause more infections associated with the use of medical devices than any other group of microorganisms (Kloos & Bannerman, 1994). *Staphylococcus epidermidis*, an inhabitant of skin and mucous membranes, is known to be an opportunistic pathogen, causing bacterial keratitis and infections in immunocompromised hosts or patients with implanted medical devices, such as intravascular and peritoneal dialysis catheters, prosthetic heart valves, or orthopaedic prostheses (Götz, 2002). CoNS other than *S. epidermidis*, such as *Staphylococcus lugdunensis* and *Staphylococcus warneri*, have been also recognized as causative agents of medical implant-associated infections. *Staphylococcus lugdunensis* is a recently described human pathogen (Ebright *et al.*, 2004) which has a tendency to cause more aggressive acute infections than is usually associated with CoNS, and therefore may be confused with *S. aureus* by a routine clinical diagnosis of infected patients and by certain *in vitro* laboratory characteristics (Ebright *et al.*, 2004). Such 'chronic polymer-associated infections' caused by *S. aureus* and CoNS are characterized by the ability of the causative microorganisms to colonize surfaces of biomaterials by adhering to their surface in biofilms-structured communities of cells encased in a self-produced polymeric matrix, an amorphous slimy material, which is loosely bound to staphylococcal cells. This ability to form biofilms is believed to make the microorganisms more resistant to administered antibiotics and to host defence mechanisms (von Eiff *et al.*, 1999).

Extracellular (EC) markers of staphylococci related to slime or biofilm production have been intensively investigated in the past decades, and *S. epidermidis* RP62A (ATCC 35984), which is considered a reference biofilm-positive strain, was used as a preferential model strain for these studies by a number of authors (Christensen *et al.*, 1990; Mack, 1999). A surface polysaccharide, common for both *S. epidermidis* and *S. aureus*, was first isolated by Tojo *et al.* (1988) from the culture supernatant of *S. epidermidis* strain RP62A. Chemical characterization of this polysaccharide, later referred to as polysaccharide intercellular adhesin (PIA), showed it to be a linear poly-*N*-acetyl-(1 → 6)-β-D-glucosamine (PNAG) (Mack *et al.*, 1996). Heilmann *et al.*

(1996) identified the biosynthetic operon for this polysaccharide adhesin and termed it *ica*. Later, a similar gene locus was identified in the clinical isolates of *S. aureus* (McKenney *et al.*, 2000). Upon correction of the certain analytical artefacts (Joyce *et al.*, 2003) and critical analysis of the data obtained for the polysaccharides isolated from the biofilms of model strains *S. epidermidis* RP62A and *S. aureus* MN8m, a conclusion has been made that the polysaccharide constituents, to which both initial attachment to abiotic surfaces and mediation of intercellular bacterial adhesion were ascribed, were all chemically PNAG (Maira-Litran *et al.*, 2004; Sadovskaya *et al*, 2005).

The second carbohydrate moiety that was characterized in a biofilm of a reference biofilm-positive strain *S. epidermidis* RP62A is EC teichoic acid (TA). While cell wall TA (CW TA) is a common component of all Gram-positive bacteria, EC TA has been discovered only in a limited number of species (Jacques *et al.*, 1979; de Boer *et al.*, 1981). We have recently established that the chemical structure of the CW and EC TAs of *S. epidermidis* RP62A is identical. They consist of (1 → 3)-linked poly(glycerol phosphate), substituted at the C-2 positions of glycerol residues with α-D-Glc, α-D-Glc*N*Ac, D-Ala and, most interestingly, α-D-Glc6Ala. The D-alanyl substitution of a sugar residue along with the poly(polyol phosphate) chain seems to be a novel structural feature for a TA (Sadovskaya *et al.*, 2004). We have also shown that the EC TA is an important component of the biofilm of the model strain *S. epidermidis* RP62A *in vitro* (Sadovskaya *et al.*, 2005).

In a recent study, we have characterized 66 strains of CoNS isolated from infected patients in Hôpital Mignot, Le Chesnay, France (Chokr *et al.*, 2005). Most of these clinical isolates were associated with orthopaedic prosthesis infections. These strains were screened for the presence of the *ica* operon, their ability to form biofilms and to produce PIA (PNAG) *in vitro*. Among these medical implant-related biofilm-positive strains, some either did not contain the *ica* locus (*ica*$^-$ strains) or did not seem to produce PIA *in vitro*, despite the presence of the *ica* locus. These data indicated that antigens other than PNAG were present in the biofilms of these strains. Furthermore, recent data indicate the presence of PNAG-independent mechanisms of biofilm formation in clinically significant *S. epidermidis* (Rohde *et al.*, 2005) and *S. aureus* (Beenken *et al.*, 2004; Fitzpatrick *et al.*, 2005; Toledo-Arana *et al.*, 2005) isolates.

In the present study, we investigated the chemical composition of the biofilms of four clinical strains of CoNS – two *S. epidermidis*, one *S. warneri*, and one *S. lugdunensis*. We have shown that their biofilms contain predominantly TA and protein components and, unlike the biofilm of the model strains *S. epidermidis* RP62A, they are not dispersed by metaperiodate, while being partially degraded by proteinase K.

Material and methods

Bacterial strains and culture conditions

Staphylococcus epidermidis RP62A (ATCC 35984) was kindly provided by Prof. Gerald Pier (Harvard Medical School, Boston, MA). The clinical isolates of *S. epidermidis* strains 392 and 495, *S. ludgunensis* 47, and *S. warneri* 446a were selected out of the 66 CoNS strains collected from infected patients hospitalized in the Mignot Hospital of Versailles, France (Chokr *et al.*, 2005). Cells were grown statically at 37 °C for 24 h in 0.7 L tryptic soy broth (TSB; Becton Dickinson, Le Pont de Claix, France) in 3 L Erlenmeyer flasks. All cultures were preliminarily inoculated with 3% (volume in volume) of 20 h preculture grown in the same medium with shaking.

Preparation of cell-associated polysaccharides and CW TAs

Crude EC matrix was isolated and fractionated essentially as previously described (Sadovskaya *et al.*, 2005). Briefly, bacterial cells were collected by centrifugation (5000 ***g***, 4 °C, 20 min), suspended in 0.9% NaCl, and sonicated on ice in a plastic container (IKASONIC sonicator, IKA Labortechnik Staufen, Germany, at 50% amplitude, cycle 0.5, 4 × 30 s) to extract the cell-associated EC material. Cells were removed by centrifugation (5000 ***g***, 15 min at 4 °C). The supernatant was further clarified by a short high-speed centrifugation (12 000 ***g***, 15 min at 4 °C) and filter-sterilized to give a crude extract, which was further concentrated using an Amicon® Ultrafiltration Cell with a 10 kDa molecular cut-off membrane (Millipore, Bedford, MA). After precipitation of proteins with the addition of trichloroacetic acid (TCA) to a concentration of 5% and their removal by high-speed centrifugation, the concentrated crude extract was applied to a Sephacryl S-300 column. Five millilitre fractions were collected and analysed for the presence of aminosugars using Elson–Morgan assay with subsequent colorimetric detection at 530 nm (Enghofer & Kress, 1979), and for the presence of neutral sugars using phenol–sulphuric acid assay and colorimetric detection at 485 nm (Dubois *et al.*, 1956). Fractions corresponding to EC TA, which gave colour reaction at both assays, were freeze dried, de-proteinated by extraction with phenol, and desalted on a Sephadex G-50 column. CW TAs were prepared from the sodium dodecyl sulphate-treated cells by TCA extraction, as described previously (Sadovskaya *et al.*, 2004). TAs were analysed by gas liquid chromatography (GLC) after depolymerization with HF, followed by acid hydrolysis, reduction, and acetylation.

Microtitre plate biofilm detachment assay

Biofilm detachment assays were carried out essentially as described in Wang *et al.* (2004). Precultures were grown

overnight in TSB with shaking. The wells of a 96-well polystyrene tissue culture-treated Nunclon microtitre plate (Nunc, Roskilde, Denmark) were filled with 200 μL aliquots of inoculum (2%), and the plate was incubated for 24 h at 37 °C without shaking. The biofilms were washed with 200 μL of 0.9% NaCl and then treated for 2 h at 37 °C with 100 μL of 10 mM sodium metaperiodate in 50 mM sodium acetate buffer (pH 4.5), or 100 μL proteinase K (Sigma, St Louis, MO, 100 μg mL^{-1} in 20 mM Tris (pH 7.5)-100 mM NaCl). Control wells were filled with an appropriate buffer. After treatment, the biofilms were washed with 200 μL of 0.9% NaCl, dried for 45 min at 55 °C, stained with 5% safranine (AES Laboratoire, Combourg, France) for 5 min, and then washed again with 0.9% NaCl. The optical density was measured using a μQuant microtitre plate reader (Bio-Tek Instruments Inc., Winvoski, VT) set to 492 nm. Statistical analysis was performed using Mann–Whitney *U*-test (http://eatworms.swmed.edu/~leon/stats/utest.cgi).

General and analytical methods

Glycerol and monosaccharides were identified by gas–liquid chromatography (GLC) on a Shimadzu GC-14 B gas chromatograph equipped with a flame ionization detector and a Zebron ZB-5 capillary column (30 m × 0.25 mm, Phenomenex, Torrance, CA), with hydrogen as carrier gas, using a temperature program 170 °C (3 min) → 260 °C at 5 °C min^{-1}. Polysaccharide samples were converted to alditol acetates by conventional methods following hydrolysis with 4 M trifluoroacetic acid at 120 °C for 2 h. Samples containing TA were depolymerized with 48% HF prior to hydrolysis.

Gel permeation chromatography was carried out on Sephacryl S-300 (1 × 90 cm) and Sephadex G-50 (1.6 × 75 cm, Pharmacia Biotech, Uppsala, Sweden) columns, eluted with water. Dialysis was performed in Spectra/Por dialysis tubing (Spectrum Laboratories Inc., Rancho Dominguez, CA) with molecular weight cut-off of 6–8 kDa against de-ionized water.

Results

Biofilm composition

The selection of the strains was based on the previously performed screening of biofilm-producing ability of 66 clinical CoNS strains (Chokr *et al.*, 2005). Using the described extraction procedure and chromatographic separation with colorimetric detection analysis (Sadovskaya *et al.*, 2005), it was possible to obtain the elution profiles of the biofilms of the individual strains, which provided preliminary information on their composition. Figure 1(a) presents a chromatographic elution profile of the crude biofilm extract of the model strain *Staphylococcus epidermidis* RP62A. As was previously shown (Sadovskaya *et al.*, 2005), PNAG was eluted as the higher molecular weight carbohydrate fraction that gave positive coloration only in Elson–Morgan aminosugar assay, while the later eluting fraction contained TA. We therefore screened elution profiles of 15 strains from our collection, which have been previously shown to produce a relatively large amount of biofilm in polystyrene microtitre wells (Chokr *et al.*, 2005), and have chosen four strains, in which the PNAG fraction was not detectable: two strains of *S. epidermidis* (392 and 495, both *ica*$^+$), *S. lugdunensis* 47 (*ica*$^-$), and *S. warneri* 446a (*ica*$^-$). Elution profiles of these strains are shown in Figs 1b–e. As can be seen, all four strains revealed only the presence of the later eluting carbohydrate-containing fraction. In order to confirm that this material indeed corresponded to EC TA, the CW TAs were isolated for all four strains and their chemical composition was compared with that established for the corresponding later-eluting carbohydrate-containing fractions.

Chemical composition of EC and CW TAs

Extracellular teichoic acids were purified from the EC extracts, and CW TAs from the sodium dodecyl sulphate-treated cells by TCA extraction, as described previously (Sadovskaya *et al.*, 2004). From the four strains studied, only the TAs of *S. lugdunensis* 47 had a composition similar to that of the model strain *S. epidermidis* RP62A and contained glycerol, glucose, and glucosamine, indicating a similar poly(glycerol phosphate) structure. The GLC analysis of the acid hydrolysate of other TAs revealed, in addition, the presence of ribitol and 1,5-anhydro-ribitol, thus indicating a more complex composition resembling that of the TA from *S. aureus* MN8m (E. Vinogradov, I. Sadovskaya, J. Li and S. Jabbouri, unpublished results). In all cases, the chemical composition of the EC TAs isolated from the biofilms was similar to that of the corresponding CW TAs.

Biofilm detachment

It has been previously shown (Wang *et al.*, 2004) that in *Escherichia coli*, where intercellular adhesion is mediated by PNAG, biofilms are disrupted by metaperiodate. If the biofilm does not contain PNAG, it should be resistant to the PNAG-degrading agents. We therefore assayed the sensitivity of the biofilms produced by the four CoNS strains to metaperiodate (HIO_4 and $NaIO_4$), known to cause the detachment of the preformed biofilms containing PNAG (Wang *et al.*, 2004). The preformed biofilm of the model strain *S. epidermidis* RP62A, which is known to contain PNAG, was used as a reference. Our results demonstrated that while the biofilm of *S. epidermidis* RP62A was partially disrupted by metaperiodate, the latter had no effect on the biofilms of the clinical strains ($P < 0.002$) (Fig. 2a).

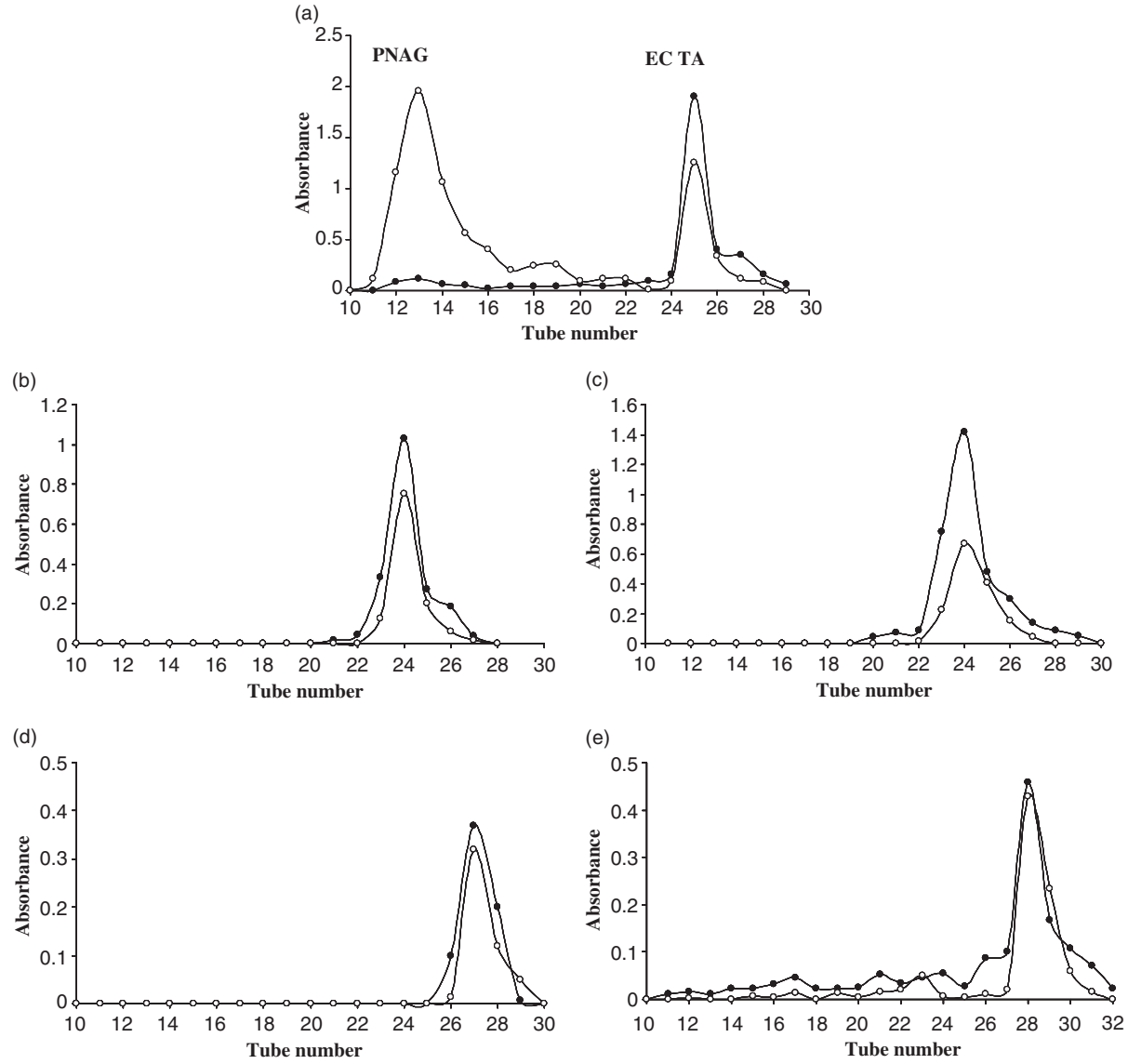

Fig. 1. Elution profiles of the extracellular extracts of *Staphylococcus epidermidis* RP62A (a), *S. epidermidis* 392 (b), *S. epidermidis* 495 (c), *Staphylococcus lugdunensis* 47 (d), and *Staphylococcus warneri* 446a (e) on Sephacryl S-300 column. Aliquots (200 μL) of each 5 mL fractions were assessed for neutral sugars (●, $OD_{485\,nm}$) and aminosugars (○, $OD_{530\,nm}$). Data correspond to 4 L of bacterial culture.

To understand the nature of macromolecules stabilizing the intercellular structure of the biofilms of the four CoNS strains, we have also treated their biofilms with proteinase K at 37 °C for 2 h. We observed a partial (from 30% to 85%) dispersal of these biofilms, while the biofilm of *S. epidermidis* RP62A remained intact (Fig. 2b).

Discussion

It has been established that coagulase-negative staphylococci and *Staphylococcus aureus* produce at the appropriate conditions biofilms that mediate adherence of bacterial cells to biomaterials and help avoid the host immune defence (Götz, 2002). The *icaADBC* locus containing the genes responsible for biosynthesis of the linear (1 → 6)-β-linked *N*-acetyl-D-glucosamine polymer was identified both in *S. aureus* and *S. epidermidis*, the major causative agents of prosthetic device-associated infections. Recent findings strongly support the assumption that this polysaccharide is a common antigen of *S. aureus* and CoNS mediating their initial attachment to the surface, as well as clumping of cells into microcolonies and larger aggregates (Maira-Litran *et al.*, 2004). However, recent reports point out to the importance of an alternative, *icaADBC*- and PNAG-independent mechanism of biofilm

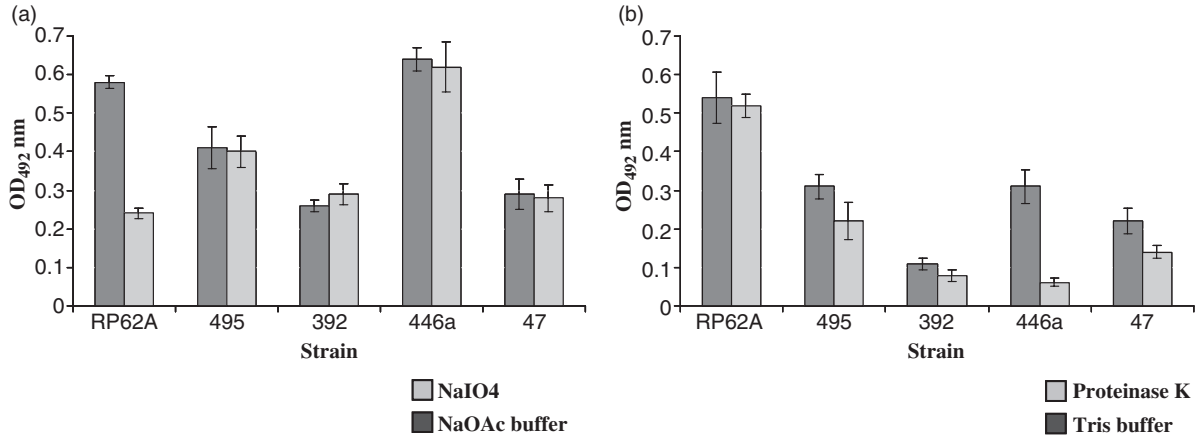

Fig. 2. Dispersal of biofilms of *Staphylococcus epidermidis* RP62A and clinical strains of coagulase-negative staphylococci by metaperiodate and proteinase K. Preformed biofilms were treated with $NaIO_4$ in NaOAc buffer (a) and proteinase K in Tris buffer (b) for 2 h at 37 °C. Control wells were filled with appropriate buffers. Average results ± standard deviations for eight wells for each strain are shown. The experiments were performed three times with similar results.

formation: involving the product of a limited proteolysis of the accumulation-associated protein Aap in *S. epidermidis* 5179 and four additional clinical *S. epidermidis* strains (Rohde *et al.*, 2005), or dependent on *arlRS* signal transduction mechanism in *S. aureus* (Toledo-Arana *et al.*, 2005). Preliminary screening of the biofilm-positive strains from our collection indicated that some *ica*$^-$ and/or PNAG$^-$ strains (determined on the basis of the immuno dot-blot assay) were nevertheless biofilm producers (Chokr *et al.*, 2005). In the present study, we analysed the chemical composition of the biofilms of four such strains, and did not find any detectable amount of PNAG.

Absence of PNAG in the biofilms of *S. lugdunensis* 47 and *S. warneri* 446a was in accordance with the absence of *ica* locus. In the *ica*$^+$ strains *S. epidermidis* 392 and 495, the *ica* genes may be inactivated by the insertion of IS*256* elements (Ziebuhr *et al.*, 1999), posttranscriptional regulations (Dobinsky *et al.*, 2003), or the action of the *icaR* repressor (Conlon *et al.*, 2002).

According to the obtained elution profiles (Fig. 1), EC TA was the main carbohydrate-containing polymer of the biofilms of the four strains studied. As in the model strain *S. epidermidis* RP62A (Hussain *et al.*, 1992; Sadovskaya *et al.*, 2004), the chemical composition of the EC TA for each strain was similar to the composition of the corresponding CW TA. This is in agreement with the fact that a large fraction of the CW TA is located in a "fluffy"-layer region beyond the CW (Neuhaus & Baddiley, 2003) and indicates that the molecules of staphylococcal TA could be released from the cell surface and become a component of the EC "slime", or a biofilm, when the cell aggregate is attached to a solid surface.

The composition of TAs of three of the four studied strains differed from that of the model strain *S. epidermidis* RP62A, pointing out to more complex structures. Establishing the detailed chemical structure of these TAs, important components of the staphylococcal biofilms, needs further investigation.

Summarizing, we have shown, by the chromatographic separation of the EC polymeric substances and their chemical analysis, that the biofilms of certain staphylococcal strains do not contain PNAG, but comprise mostly TA and proteins. Accordingly, the biofilms of these strains were not sensitive to the action of metaperiodate, a PNAG-degrading agent. On the other hand, they were disrupted by proteinase K indicating that macromolecules that stabilized their biofilms were not PNAG but rather protein components.

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