Competence-specific Autolysis in *Streptococcus sanguis*

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Streptococcus sanguis strain Wicky activated to competence for genetic transformation is known to undergo a rapid decrease in optical density upon transfer to an alkaline buffer containing reducing agents. We studied the mechanism of this autolysis-like process and made the following observations. The process was specific because preincubation of the competence inducing factor with a specific inactivating protein prevented both cellular lysis and acquisition of competence for genetic transformation. The optical density decrease of competent bacteria involved the release of a large fraction of intracellular protein, RNA and lipid. However, no hydrolysis of phospholipid and no degradation of cell wall polymers including peptidoglycan could be detected. No peptidoglycan hydrolase activity capable of degrading radiolabelled *S. sanguis* cell walls was detected in unfractionated *S. sanguis* extracts. It is suggested that autolysis of competent *S. sanguis* involves the activity of a novel type of murein hydrolase that introduces only a limited number of bond breaks into the peptidoglycan.

INTRODUCTION

The widespread occurrence of cell wall degrading enzymes, autolysins, among bacteria and the phenomenon of autolysis have led to much speculation about the possible physiological roles and cellular control of these enzymes (Shockman *et al.*, 1974; Tomasz & Höltje, 1977; Tomasz, 1983). Such a control mechanism would be essential to maintain cellular integrity during those processes which are assumed to require localized and/or transient autolytic activity, i.e. cell wall turnover, separation of daughter cells following cell division (Rogers, 1970, 1981), and competence to undergo genetic transformation (Tomasz, 1969; Young *et al.*, 1964; Akrigg *et al.*, 1967). It has been proposed that one of the early events following interaction of cells with the competence (inducing) factors (Tomasz & Hotchkiss, 1964; Pakula, 1965) in both the pneumococcal and streptococcal transformation systems is the triggering of limited murein hydrolase activity at the cell equator. The hydrolase, presumably rupturing relatively few covalent bonds to expose DNA binding sites, would not affect the structural integrity of the cell. On the other hand, lysis and rupture of the cell wall, which has already been weakened, may occur when these bacteria are exposed to conditions that promote autolysis such as exposure to hypertonic medium (Seto & Tomasz, 1975) or postincubation in special media (Ranhand *et al.*, 1971; Ranhand, 1973). The existence of competence-related lysis in *S. sanguis* is of particular interest since many strains of this bacterium (including strain Wicky) are naturally occurring penicillin-tolerant bacteria which do not undergo lysis during treatment with cell wall inhibitors (Horne & Tomasz, 1980), and testing of *S. sanguis* extracts for murein hydrolase activity has yielded only negative results (Horne & Tomasz, 1977). In this communication we describe studies on the autolysis of competent *S. sanguis* using bacteria selectively labelled with radioactive tracers in various cell components. Our data support the suggestion of Ranhand (1973) that the autolysis of competent *S. sanguis* involves breakage of a limited number of covalent bonds in the peptidoglycan.

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Abbreviations: AIF, activation inhibition factor; CF, competence factor; LTA, lipoteichoic acid; PG, peptidoglycan; PYG, proteose-peptone/yeast extract medium.

METHODS

Bacterial cultures. *Streptococcus sanguis* strain Wicky was used in these studies since the bacteria are noncompetent for genetic transformation unless treated with exogenous competence factor (CF; Pakula, 1965). Stock cultures were prepared in brain heart infusion broth (16 h, 37 °C; Difco). The overnight culture was diluted 50-fold into an enriched chemically defined medium (Cden; Tomasz, 1966) buffered at pH 7.6 with 0.05 M-sodium phosphate buffer.

Reagents. Benzylpenicillin was purchased from Eli Lilly, Indianapolis, Ind., USA. The *Chalaropsis* muramidase was prepared by the method of Hash & Rothlauf (1967). All other reagents were from commercial sources.

Transformation procedures. Cell-free extract derived from competent cultures of the related *S. sanguis* Challis strain grown in the proteose-peptone/yeast extract medium (PYG; Perry, 1968) was used as a source of CF; the cell-free filtrates were heated at 85 °C for 15 min to remove a bacteriocin as described previously (Schlegel & Slade, 1973; Horne & Perry, 1974). Transforming DNA was prepared from a mutant of *S. sanguis* Challis that was resistant to 5 mg streptomycin ml⁻¹. The DNA was isolated according to the modified method of Young & Spizizen (1961) using the C-phage associated lysin to rupture the cells (Fischetti *et al.*, 1971).

Competence was induced in cultures of *S. sanguis* Wicky by adding the CF-containing filtrates (20%, by vol.). CF was added to *S. sanguis* Wicky growing exponentially in the Cden medium at a cell density of 2×10^7 c.f.u. ml⁻¹ and competence developed after incubating the cultures at 37 °C for 30 min. Control noncompetent cultures were prepared by adding PYG medium in place of CF. Samples of 1 ml were removed and exposed to the transforming DNA (5 µg ml⁻¹) and the number of transformants was determined as described elsewhere (Horne & Perry, 1974). The purification of the putative pneumococcal CF receptor, also known as the activation inhibitory factor (AIF), was as described previously (Horne *et al.*, 1977b).

Biosynthetic labelling of the macromolecular components of cells. The radioactively-labelled precursors used in these studies were: [³H]glycerol (5 Ci mmol⁻¹; 1 µCi and 10 µg per ml of growth medium); [³H]acetic acid (sodium salt; 125 mCi mmol⁻¹; 10 µCi and 6.8 µg ml⁻¹); [6-³H]uracil (20 Ci mmol⁻¹; 2.5 µCi and 10 µg ml⁻¹); L-[4,5-³H]lysine (60 Ci mmol⁻¹; 1 µCi and 9 µg ml⁻¹); and L-[methyl-³H]methionine (10 Ci mmol⁻¹; 1 µCi and 15 µg ml⁻¹) [1 Ci = 37 GBq]. All of these precursors were purchased from New England Nuclear. N-Acetyl-D-[1-³H]glucosamine ([³H]GlcNAc; 4.7 Ci mmol⁻¹; 2 µCi and 0.1 µg ml⁻¹) was obtained from Amersham. Unless otherwise stated in the text, the radioactive precursor was added at a cell density of approximately 5×10^6 c.f.u. ml⁻¹ and kept in the medium throughout the period of competence development.

The amount of radioactivity lost from the cells during autolysis was measured as described previously (Horne & Tomasz, 1977). Cell-free supernatant fluids were prepared by removing the bacteria by centrifugation in a microcentrifuge (12000 g, 10 min). The label that was incorporated into macromolecular material was measured following precipitation with ice cold 10% (w/v) TCA. The amount of labelled lysine or GlcNAc incorporated into the peptidoglycan (PG) was assayed by the modified Park-Hancock fractionation procedure (Park & Hancock, 1960; Horne & Perry, 1974).

Biosynthetic labelling of cell walls. [³H]GlcNAc or [³H]lysine was added to 20 ml of an exponentially growing culture at a cell density of 10^7 c.f.u. ml⁻¹ and growth was continued for 2 h. The bacteria were harvested by centrifugation and washed three times with distilled water. They were combined with cells from 200 ml of nonlabelled culture and crude cell walls were isolated (Mosser & Tomasz, 1970). Purified cell walls were obtained by extracting the crude cell walls with 2% (w/v) SDS at 80 °C for 30 min followed by extensive washing.

Determination of peptidoglycan digestion. Various preparations of labelled cell walls were incubated with autolysed *S. sanguis* Wicky or with a heterologous murein hydrolase, the *Chalaropsis* muramidase. Cells were removed from crude autolysates by centrifugation (10000 g, 10 min). The test was done as follows: 10 µl of a suspension of labelled cell walls (0.3 to 1×10^4 c.p.m.) were mixed with 250 µl of autolysate or with 10 µl of the muramidase (1 mg ml⁻¹) in 240 µl sodium acetate buffer, pH 4.8 for 24 h at 37 °C. After incubation, 20 µl of 30% (w/v) formaldehyde and 20 µl of 4% (w/v) bovine serum albumin solution were added and the undigested walls were pelleted by centrifugation at 12000 g (10 min). Radioactivity released from the cell walls was measured by dissolving 200 µl samples in Biofluor scintillation fluid (Beckman) and counting in a Mark II scintillation spectrometer (Nuclear Chicago).

Autolysis of *S. sanguis* Wicky. The lysing buffer 0.05 M-Tris(hydroxymethyl)methylaminopropanylsulphonic acid (TAPS)/0.02 M-2-mercaptoethanol/0.01 M-EDTA, pH 9.2 of Ranhand *et al.* (1971) was used. Competent and noncompetent cells were pelleted by centrifugation (4000 g, 10 min, 4 °C), washed twice in cold 0.15 M-NaCl and resuspended in the original volume of distilled water. To initiate autolysis, the bacteria were diluted 1:10 into the lysing buffer and incubated at 37 °C. OD₅₅₀ was determined at intervals (1 cm cuvettes). In some experiments, the crude autolysates were used to lyse suspensions of noncompetent cells in a manner similar to the assay for solubilization of labelled cell walls.

Lipid extraction and TLC. The bacterial lipids were extracted from [³H]acetate-labelled cells by the method of Ames (1968). TLC was done using the solvent system chloroform/methanol/water (65:25:4, by vol.) to develop the chromatograms (Horne *et al.*, 1977a). Bands 0.5 cm wide were scraped into scintillation vials for measurement of radioactivity.

SDS-PAGE. Cell-free extracts obtained from [^3H]glycerol-labelled bacteria after autolysis (2 h, 37 °C) were dialysed and concentrated. The material was then applied to a 10% polyacrylamide gel. The method of Weber & Osborn (1969) was used for electrophoresis in the presence of SDS. The amount of radioactivity was determined in 1.2 mm slices of the gels as described earlier (Horne & Tomasz, 1977).

RESULTS

Autolysis of competent versus noncompetent S. sanguis Wicky

Fig. 1 (a) illustrates autolysis of competent cells when resuspended in lysing buffer. Autolysis, detected as a decrease in the optical density of the cell suspension with time, appeared to be specific for the competent state. The decline in the optical density of noncompetent cells was much lower than that observed with the competent bacteria. The simultaneous addition of the pneumococcal competence activation inhibitory factor (AIF) along with CF prevented autolysis as well as inhibiting the development of competence.

AIF decreased the number of transformants by more than two orders of magnitude compared with cultures which were treated with CF alone (Fig. 1a, inset). Pretreatment with penicillin, which is known to trigger autolytic activity in many bacteria (Tomasz, 1979), did not stimulate autolysis of noncompetent cells (Fig. 1a).

Loss of radiolabelled protein and RNA during autolysis

There was a substantial release of intracellular, TCA-precipitable components during the course of autolysis in the competent cells (Fig. 1b). Approximately 18% of the total protein was detected in the cell-free supernatant fluids after 30 min of incubation in the buffer (shown here with [^3H]methionine-labelled bacteria). In the same time period only about 1% of the labelled protein was lost from noncompetent cells. Similar results were obtained for RNA labelled with [^3H]uracil. After 2 h in lysing buffer, 30% of labelled RNA was detected outside the competent cells compared with only about 1% in the noncompetent bacteria. The total TCA-precipitable radioactivity declined during incubation in the lysing buffer; however, the decrease in precipitable counts was virtually identical in both types of cells (not shown).

Noncompetent cells lysed following suspension in a crude autolysate derived from competent cells. The loss of the intracellular markers occurred to about the same extent (data not shown). This finding is in accord with the report of Ranhand *et al.* (1971) who assumed that a soluble component was released by the autolysed bacteria and that the component was probably an enzyme since it was heat-labile.

Although the amount of the total cell RNA and protein lost from the cells was considerable, it was certainly not as much as the quantities released from bacteria undergoing autolysis due to extensive cell wall degradation. Ranhand *et al.* (1971) indicated that much of the cell wall remained intact and that only a limited amount of damage could be observed at the equatorial zone of the cells. We therefore attempted to determine if any cell wall-specific material was solubilized during autolysis.

Assay for peptidoglycan degradation during autolysis of competent cells

In order to detect possible cell wall damage, we utilized either [^3H]GlcNAc or [^3H]lysine as precursors of cell wall polymers. The former compound has been shown to be a specific label for the cell walls, being incorporated into PG and cell wall polysaccharide about equally (unpublished observations; Horne & Perry, 1974). Lysine is incorporated into both protein (80%) and PG (20%) (Horne & Perry, 1974). In these experiments, the bacteria were either continuously labelled with the precursor as described in Methods or subjected to a 15 min pulse followed by a chase period of one doubling time in unlabelled medium. During autolysis very little if any label was lost from cells labelled with [^3H]GlcNAc or from the PG of cells labelled with [^3H]lysine if the cells were continuously labelled (Table 1, continuous labelling expts 1, 2 and 3) or pulse-chased with [^3H]GlcNAc (Table 1, expts 4 and 5). The retention of the label suggested that the bulk of the cell wall remained intact during autolysis. In order to detect any selective degradation of newly made cell wall material, the bacteria were pulsed with GlcNAc during the last 15 min of competence induction before being placed in the lysing buffer. No digestion of the nascent cell wall material occurred (Table 1, expts 6 and 7).

Fig. 1. Autolysis and release of intracellular macromolecular material from *S. sanguis* Wicky. Cells grown in Cden, pH 7.6, to a cell density of 2×10^7 c.f.u. ml⁻¹ were treated with 20% (v/v) CF for 30 min at 37 °C for development of competence. Control, noncompetent cultures were treated with the same volume of PYG or with benzylpenicillin (0.5 µg ml⁻¹) for 45 min at 37 °C. (a) Autolysis of competent and noncompetent cell suspensions in lysing buffer. CF-treated competent cells (●, solid lines); untreated noncompetent cells (○, dashed lines); cells treated simultaneously with CF and 1.4 µg pneumococcal AIF ml⁻¹ (▲, dashed lines); and cells pretreated with penicillin (■, dashed lines). The inset indicates the number of transformants per ml obtained after 1 ml of each culture was removed after competence induction and exposed to transforming DNA as described in Methods. (b) Release of intracellular macromolecular components during autolysis. Cells were labelled with [3H]methionine ([3H]Met) or with [3H]uracil ([3H]Ura) before and during competence induction as described in Methods and subjected to autolysis. The cell-free TCA-precipitable c.p.m. were detected as described in the text. Competent cells (●, solid lines); noncompetent cells (○, dashed lines). The percentage of the total TCA-precipitable material set free in competent versus noncompetent cells was as follows: 18% versus 1% with [3H]methionine-labelled cells and 30% versus 0.9% in [3H]uracil-labelled cells.

Assay for peptidoglycan hydrolase activity in the autolysate of competent cells

Purified cell walls radiolabelled with [3H]lysine or [3H]GlcNAc were treated at 37 °C with cell-free extracts derived from autolysed competent cells. The release of radioactivity from the labelled cell walls in autolysate-treated samples was indistinguishable from that in control samples even after the prolonged incubation period (18 h; Table 2). In contrast, the *Chalaropsis* muramidase digested a major portion of the cell wall-associated label.

Assay for phospholipase activity during autolysis of competent cells

The possibility that autolysis was mediated by damage to the cell membrane rather than to PG was considered. The possible involvement of a phospholipase associated with autolysis was

Table 1. *Lack of peptidoglycan degradation during autolysis of competent cells*

Unless otherwise stated, bacteria were labelled with the radioactive precursors and subsequently fractionated by the standard procedures described in Methods.

Experiment	Fraction	TCA-insoluble c.p.m. per 200 μ l	
		Competent cells	Noncompetent cells
1. Continuous labelling [3 H]GlcNAc	Total	5070	4425
	Supernatant fluids	87	63
2. Continuous labelling [3 H]lysine	Total (A)	6900	6090
	Total PG† (B)	2298	1952
	Cell-associated PG (C)	2053	1367
	Cell-free PG (B - C)	245	585
3. As in 2	Total (A)	5731	6085
	Total PG (B)	1738	1912
	Cell-associated PG (C)	1690	1610
	Cell-free PG (B - C)	48	302
4. Pulse-chase* [3 H]GlcNAc	Total	1330	1142
	Supernatant fluids	72	75
5. As in 4	Total	1627	1450
	Supernatant fluids	30	9
6. Pulse† [3 H]GlcNAc	Total	7560	8730
	Supernatant fluids	176	93
7. As in 6	Total	12250	13536
	Supernatant fluids	478	113

* Bacteria were labelled with 2 μ Ci [3 H]GlcNAc ml $^{-1}$ at a cell density of 10^7 c.f.u. ml $^{-1}$ for 15 min at 37 °C. The cells were then harvested by centrifugation, washed with medium three times and resuspended in prewarmed nonradioactive medium and incubated at 37 °C for one generation time until competence induction. Competence was induced as described in Methods.

† Competent cells (cell density 3×10^7 c.f.u. ml $^{-1}$) and noncompetent cells (see Methods) were labelled with [3 H]GlcNAc (2 μ Ci ml $^{-1}$) for 15 min at 37 °C. The cells were then harvested and autolysed as described in Methods.

‡ PG, peptidoglycan. Incorporation of the precursors into peptidoglycan was as described by Horne & Perry (1974).

Table 2. *Assay for peptidoglycan hydrolase activity in the autolysate of competent cells*

The labelled cell walls were incubated with the preparations in the appropriate buffer for 18 h at 37 °C and 200 μ l samples were examined. Assay for the solubilization of the cell walls is described in Methods.

Label	Treatment	Crude cell walls		Purified cell walls	
		Total c.p.m.	Released c.p.m.*	Total c.p.m.	Released c.p.m.*
[3 H]Lysine	None	9000	488 (5.4)	5000	60 (1.2)
	Muramidase	2600	1700 (65)	2200	1728 (78)
	Autolysate	9000	492 (5.5)	5000	121 (2.4)
[3 H]GlcNAc	Autolysate	6000	124 (2.0)	6000	78 (1.0)

* Percentage of total in parentheses.

investigated. The lipids of competent and noncompetent cells were labelled with [3 H]acetate (95% incorporated into lipid constituents; Horne *et al.*, 1977a) before autolysis. Following autolysis of the competent cells, the total lipid was extracted from both competent and noncompetent cell suspensions and analysed by TLC. No alteration in the TLC patterns could be detected between the competent and noncompetent extracts (Fig. 2). Furthermore, no evidence of phospholipase activity was detected in either type of cell suspension. There was no evidence for a 'new' band migrating slower than peak I representing the phospholipids of *S. sanguis* strain Wicky, presumably phosphatidyl glycerol and cardiolipin (Horne *et al.*, 1977a), nor was there any increase in the height of peak IV (neutral lipids) following incubation in lysing buffer (data not shown).

Fig. 2. TLC of [^3H]acetate-labelled lipids extracted from autolysed *S. sanguis* Wicky. Lipid extracts from cell suspensions were developed in chloroform/methanol/water and the radioactivity in 0.5 cm bands was measured as described in the text. The arrows represent the origin (O) and the solvent front (F). The four major peaks have been identified: I represents two phospholipids (PL1 and PL2); II and III represent glycolipids (GL1 and GL2) and IV represents neutral lipid (NL). Lipids of competent cells (●, solid lines) and noncompetent cells (○, dashed lines).

Analysis of [^3H]glycerol-containing cell components released during autolysis

A significant portion of [^3H]glycerol-labelled materials was lost from competent cells (60% of total radioactivity) and a lesser amount from noncompetent cells (26% of total) during autolysis (2 h, 37 °C). The radioactive precursor has been found to be incorporated into the membrane-associated polyglycerophosphate type of lipoteichoic acid (LTA) and the lipids in *S. sanguis* Wicky (Horne & Tomasz, 1977). The material lost from the competent cells was analysed by SDS-PAGE (Fig. 3). The material containing [^3H]glycerol was separated into two major types of constituents: region I, which represented the polyglycerophosphate polymer (deacylated and acylated forms of the polymer) and region II, which represented the lipids. About 15% of the total radioactivity applied to the gel was detected in peak I and 81% in peak II. Although the amount of released label was less in noncompetent cells, virtually the same pattern was obtained when the material was analysed by SDS-PAGE. [^3H]Glycerol-labelled material derived from crude cell wall-membrane complexes analysed by the same methods gave a somewhat similar pattern: about 25% of the label was associated with region I and 70% with region II (data not shown). The electrophoretic profile of the [^3H]glycerol-labelled components released during autolysis of competent cells was quite different from that of similarly labelled material secreted during treatment with benzylpenicillin. In the latter case, there appeared to be a somewhat selective release of the LTA as over 60% of the secreted material was associated with the slower migrating material and only 35% was detected in the lipid region (Horne & Tomasz, 1977). The data presented here suggest the loss of fragments of the plasma membrane during autolysis and no enrichment for LTA over its relative proportion among the glycerol-containing components found within the cells.

DISCUSSION

Ranhand *et al.* (1971) observed that *S. sanguis* made competent for genetic transformation by treatment with CF lysed (as judged by the decrease of optical density) upon transfer of the cells

Fig. 3. SDS-PAGE of glycerol-labelled material released from competent cells after 2 h at 37 °C in autolysis buffer. The bacteria and cell debris were removed by centrifugation from a 10 ml competent culture, labelled with [³H]glycerol, and the resulting supernatant fluids were collected. The amount of labelled material released from competent cells amounted to 60% of the total versus 26% released in noncompetent cells. After extensive dialysis the material was subjected to SDS-PAGE, and the gels were sliced and measured for radioactivity as described in Methods. Two major types of components are indicated by the horizontal bars: I, LTA; II, the lipids. The arrow represents the position of the tracking dye.

to an alkaline buffer containing reducing agents. Susceptibility to lysis appeared to be specifically related to the competent state since only strains capable of genetic transformation exhibited lysis and conditions preventing induction of competence (e.g. inhibition of protein synthesis) also blocked lysis (Ranhand *et al.*, 1971). We have confirmed and further extended the evidence for this specificity. Pretreatment with penicillin (instead of CF) did not induce autolysis of cultures. Low concentration of the AIF, a specific inhibitor and putative cellular receptor of CF (Horne *et al.*, 1977*b*), inhibited both induction of competence and autolysis. Finally, cultures in which the level of competence was low also displayed low degrees of autolysis (undocumented observations).

We tried to define better the nature and mechanism of the decline in optical density of such bacterial suspensions. The possibility that lysis was the result of structural damage to the plasma membrane, followed by the escape of cytoplasmic material, was considered. Activation of a phospholipase during development of competence could conceivably be responsible for the observed autolysis. However, no phospholipase activity could be observed and there was no detectable qualitative or quantitative change in the phospholipids of autolysing cells (see Fig. 2). Similarly, no loss of PG could be detected from cells which were grown for several generations with radioactively labelled cell wall precursors and then allowed to lyse in the alkaline buffer. The possibility that lysis involved cell wall degradation restricted to the newly labelled portion of the PG was also tested. However, PG which had been synthesized just before autolysis during a short pulse with the radioactively labelled GlcNAc was not solubilized. Attempts to demonstrate murein hydrolase activity *in vitro* in cell-free preparations derived from autolysed cells and using radiolabelled strain Wicky cell walls as substrate were likewise negative, even though such cell-free autolysates did in fact lyse intact noncompetent cells.

Our experiments with radioactively labelled bacteria indicate that the decrease in the optical density of competent cultures was accompanied by a parallel release of a substantial fraction of

protein, nucleic acid and lipid label. This process differed from the secretion of LTA and other material previously described in penicillin-treated *S. sanguis* (Horne & Tomasz, 1979). While the penicillin-induced secretion was restricted to cell surface components (Horne & Tomasz, 1977), the material lost during lysis of competent cells included intracytoplasmic protein and nucleic acid and the high degree of selectivity for LTA-release characteristic of the penicillin-treated cells was not apparent during lysis of competent cells.

We interpret the escape of cytoplasmic markers as an indication of structural damage to the cell wall. This interpretation is in accord with that of Ranhand *et al.* (1971) who could only demonstrate limited disruption of the cell surface in electronmicroscopic thin sections of lysing *S. sanguis*. There are two intriguing aspects to this hydrolase activity. First, it seems to be limited enough not to cause solubilization of wall polymers. A mechanistically analogous cell wall 'nicking' has been proposed as the cause of 'non-lytic death' in certain groups of streptococci during treatment with cell wall inhibitors (Horne & Tomasz, 1977; Tomasz, 1979).

A second interesting aspect of the autolysis is the apparent specific relationship to the competent state. It is interesting that lysis of both the competent *S. sanguis* and the pneumococcus seems to occur by wall disruption at the cellular equator. This area also represents the growth zone of cell wall (Briles & Tomasz, 1970; Cole, 1965) and it has been suggested that the uptake of DNA molecules may also occur within this part of the bacterial surface (Tomasz *et al.*, 1971). The nature of cell wall hydrolysing activity operating at this vital and dynamic surface site remains to be identified.

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