Mode of Action of Linenscin OC2 against *Listeria innocua*

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Linenscin OC2 is a small hydrophobic substance produced by the orange cheese coryneform bacterium *Brevibacterium linens* OC2. Linenscin OC2 inhibits growth of gram-negative bacteria with an altered outer membrane permeability and gram-positive bacteria. It is also able to lyse eucaryotic cells. The mode of action of linenscin OC2 on the *Listeria innocua* cytoplasmic membrane and the effects of environmental parameters were investigated. Addition of low doses of linenscin OC2 resulted in an immediate perturbation of the permeability properties of the cytoplasmic membrane and of the bacterial energetic state. Linenscin OC2 induced a loss of cytoplasmic potassium, depolarization of the cytoplasmic membrane, complete hydrolysis of internal ATP, efflux of inorganic phosphate, and transient increase in oxygen consumption. Potassium loss occurred in the absence of a proton motive force and was severely reduced at low temperatures, presumably as a result of increased ordering of the lipid hydrocarbon chains of the cytoplasmic membrane. We propose that linenscin OC2 interacts with the cytoplasmic membrane and that the permeability changes observed at low doses reflect the formation of pore-like structures in this membrane.

**Listeria monocytogenes** is a food-borne pathogen (6, 21) able to grow under various environmental conditions (17), and it often contaminates dairy products (4, 17, 20). Of all dairy products, cheeses have most frequently been found to be contaminated with *Listeria* and associated with human diseases. Even when cheeses are produced from pasteurized milk, *Listeria* contamination can occur during their manufacture. This risk is important for soft surface-ripened cheeses, especially with white-mold and red-shear surfaces (30).

*Brevibacterium linens* is an orange coryneform bacterium used in red-shear cheese ripening. *B. linens* OC2 was isolated from the surface of the cheese Gruyère de Comté (27) as a strain which exhibited antagonistic action against *L. monocytogenes*. This strain produces an antibacterial substance named linenscin OC2, which occurs in large aggregates in the native state and has been described as a 1,200-Da hydrophobic peptide containing about 10% charged amino acids (24). Two other antibacterial substances, linencin A and linocin M18, have been previously purified from *B. linens* strains (22, 29). Linencin A is a 95-kDa protein and is only active against *B. linens* strains (22), whereas linocin M18 is a 31-kDa protein and is active against *Listeria* spp. and other gram-positive bacteria (29). It is therefore of interest to explore the potential use of *B. linens* strains which inhibit growth of *Listeria* spp. in red-shear surface-ripened cheese production.

We have previously studied the antibacterial and hemolytic activities of linenscin OC2 (7). Linenscin OC2 inhibited growth of all gram-positive bacteria tested, but it was inactive against gram-negative bacterium, *Saccharomyces cerevisiae*, and mold strains (7, 24). However, linenscin OC2 became active against gram-negative bacteria upon alteration of the outer membrane permeability barrier (7). At high doses and in a complete medium, the effect of linenscin OC2 was bacteriolytic on *Listeria innocua*. Bacteriostasis was observed with low doses of linenscin OC2, and linenscin OC2 inhibited peptidoglycan biosynthesis at some early step upstream from the UDP-N-acetyl-glucosamine synthesis. Linenscin OC2 was also able to lyse sheep erythrocytes (7).

Our initial results suggested a common mode of action of linenscin OC2 on procaryotic and eucaryotic cells and that the cytoplasmic membrane might be the primary target of linenscin OC2. In this study, we have investigated the effects of linenscin OC2 on the permeability properties of the cytoplasmic membrane of *L. innocua* and on different bioenergetic parameters.

**MATERIALS AND METHODS**

**Preparation of semi-pure linenscin OC2.** *B. linens* OC2 was grown in a semidefined medium as previously described (7). Linenscin OC2 activity was located in the foam which was collected and lyophilized. The concentrate obtained was dissolved and fractionated by size exclusion chromatography (Toyopearl HW-65F; Merck) in 10 mM Tris-HCl (pH 8.0). Active fractions (large aggregates of native linenscin OC2 eluted in the void volume) were filter sterilized and stored at 4°C.

**Antimicrobial assays of linenscin OC2.** *L. innocua* CIP8811 (Institut Pasteur, Paris, France) was used as the indicator strain. Antimicrobial activity was quantified as previously described (7) by the serial twofold dilution assay described by Mayr-Hartling et al. (25). Antimicrobial activity was defined as the reciprocal of the highest dilution yielding a definite zone of growth inhibition of *L. innocua* and expressed as activity units (AU) per milliliter. Linenscin OC2 doses were expressed as AU per milligram of *L. innocua* (dry weight) (1 ml of culture with an absorbance at 570 nm of 2 led to 0.97 mg of *L. innocua* [dry weight]).

**Growth conditions and assay medium.** *L. innocua* CIP8811 was grown in tryptic soy broth (Difco) plus 0.6% yeast extract (Difco) (TSBYE) at 37°C in a rotary shaker. Exponential-phase cells were harvested (A570 of 2) and resuspended at room temperature in 10 mM HEPES buffer (pH 6.5) containing 150 mM NaCl. Cell suspensions (A570 of 20) were kept at room temperature and used within 3 h. Cells were energized before use by addition of 0.4% (wt/vol) glucose and incubation at 37°C in a rotary shaker. Cell concentrations were expressed as milligrams of *L. innocua* (dry weight) per milliliter. All assays were performed in this medium unless indicated otherwise.

**Potassium efflux measurements.** The potassium efflux was monitored by measuring the extracellular potassium concentration with a potassium/valinomycin-selective electrode (Radiometer; 4-s resolution time) as previously described (1). Cells (0.49 mg ml−1) were incubated at 37°C in the assay medium containing 0.6 mM KCl unless indicated otherwise. The total K+ content of bacteria was...
estimated after K$^+$ was released from the cells by treatment with an excess dose of linenscin OC2 (50 AU mg$^{-1}$) and was expressed in nanomoles per milligram of cells.

**Measurement of the membrane potential (ΔΨ).** The transmembrane electrical potential was determined from the accumulation of [3H]tetraphenylphosphonium ion ([3H]TPP$^+$) (3.7 MBq nmol$^{-1}$; Amersham) (8). Cells (0.97 mg/ml$^{-1}$) were incubated at 37°C in the assay medium containing 10 μM [3H]TPP$^+$. Linenscin OC2 was added after 10 min of incubation. At appropriate time intervals aliquots of 100 μl were taken (in triplicate), filtered on Whatman glass microfiber filters (GF/F), and washed twice with 4 ml of the assay medium. [3H]TPP$^+$ uptake was corrected for nonspecific binding by subtracting a blank obtained under identical conditions, except that the cells were pretreated with the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP [Sigma]; final concentration, 10 μM) before the addition of [3H]TPP$^+$. Oxygen consumption measurements. Oxygen consumption of cells was monitored polarographically with a Clark-type electrode connected to a Gilson oxicgraph (14). Measurements were carried out at 37°C on cells (0.49 mg ml$^{-1}$) incubated in the assay medium unless indicated otherwise.

**Determination of intracellular and extracellular ATP concentrations.** Cells (0.49 mg ml$^{-1}$) were incubated at 37°C in the assay medium. To determine the total (intracellular and extracellular) ATP concentration, 20-μl aliquots of cell suspension were taken at given times and mixed with 80 μl of dimethyl sulfoxide (DMSO); after 30 s at room temperature, the suspension was diluted with 4.9 ml of ice-cold water. The total ATP concentration was determined on 0.1 ml of this dilution with the Boehringer ATP Bioluminescence CLS kit and a Lumac Lumitron (DMSO); after 30 s at room temperature, the suspension was diluted with 4.9 ml of the assay medium.

**RESULTS**

Effects of linenscin OC2 on the viability of *L. innocua* in a salt medium. The effect of linenscin OC2 has been previously studied on growing cells in TSBYE complete medium (7). It was found to be dose dependent, being bacteriostatic at low doses (130 to 850 AU mg$^{-1}$) and bactericidal and bacteriolytic at high doses (≥3,600 AU mg$^{-1}$). The effect of linenscin OC2 was investigated in assay medium (10 mM HEPES [pH 6.5], 150 mM NaCl) containing 0.4% glucose, which was the medium consistently used in this study (Fig. 1). The viability of *L. innocua* cells was only slightly affected upon a 60-min incubation in the assay medium with low doses of linenscin OC2 (≥155 AU mg$^{-1}$). Under these conditions we could not observe any change in cell absorbance. Linenscin OC2 was bactericidal at higher doses (≥225 AU mg$^{-1}$), and a decrease in cell absorbance was observed at even greater doses (≥5,400 AU mg$^{-1}$) (data not shown). Similar results were obtained regardless of the pH of the assay medium, ranging from 6.5 to 7.5 (Fig. 1).

Linenscin OC2-induced K$^+$ efflux from *L. innocua* cells. Freshly prepared *L. innocua* cells contain ca. 600 nmol of K$^+$ mg$^{-1}$. *L. innocua* is closely related to *L. monocytogenes* (15), so it is reasonable to consider the two species to have the same cytoplasmic volume. Taking into account the cytoplasmic volume of 1 μl of cells (dry weight)$^{-1}$ determined for *L. monocytogenes* (2), this corresponds to a cytoplasmic K$^+$ concentration of ca. 600 mM. No loss of K$^+$ was observed when cells were kept concentrated at room temperature, and cells did not accumulate K$^+$ upon addition of glucose (0.4%) and KCl (up to 1 mM). Addition of linenscin OC2 (20 AU mg$^{-1}$) to cells incubated at 37°C resulted in an immediate and rapid efflux of cytoplasmic K$^+$ (initial rate, 700 nmol min$^{-1}$ mg$^{-1}$) such that the cells lost all K$^+$ in approximately 100 s (Fig. 2A). The initial rate of K$^+$ efflux increased with increasing doses of linenscin OC2 (Fig. 2B), and total K$^+$ efflux was observed for linenscin OC2 doses as low as 10 AU mg$^{-1}$. The lag time preceding the K$^+$ efflux was a few seconds regardless of the dose of linenscin OC2 (data not shown).

At a high ionic strength (150 mM NaCl), linenscin OC2 induced K$^+$ efflux for pH values of the medium ranging from 5.5 to 8.5, but the highest rates of efflux were measured at acidic pHs (data not shown). For a given pH value (6.5), the initial rate of K$^+$ efflux did not vary significantly with the ionic strength of the medium, ranging from 25 to 250 mM NaCl (data not shown).

**ΔΨ changes induced by linenscin OC2.** Energized *L. innocua* cells retain a ΔΨ of 118 mV (negative inside) at pH 6.5, a value in agreement with those determined for *L. monocytogenes* (2) and other *Listeria* spp. (9, 12). The addition of linenscin OC2 (20 AU mg$^{-1}$) resulted in a dissipation of the membrane potential in less than 2 min (Fig. 3A). Figure 3B represents the effect of increasing doses of linenscin OC2 on the membrane potential measured after 5 min of incubation. A ΔΨ of ~30 mV was the lowest limit measured regardless of the linenscin OC2 dose applied. However, ΔΨ measurements at low transmembrane potentials rely strongly upon corrections for nonspecific binding of the ΔΨ probe, which in turn depends on the way the cells are deenergized (23). It is therefore reasonable to consider that linenscin OC2 fully dissipates membrane potential.

Respiratory activity of linenscin OC2-treated *L. innocua* cells. The oxygen consumption of energized cells was ca. 25 nmol min$^{-1}$ mg$^{-1}$. The addition of linenscin OC2 (≥7 AU mg$^{-1}$ and up to 40 AU mg$^{-1}$) resulted in an immediate increase in oxygen consumption (up to 1.6-fold). However, this increase was only transient: the period over which it occurred decreased with increasing linenscin OC2 doses, lasting no more than 2.5 min. Oxygen consumption ceased, although oxygen was still available in the incubation medium (data not shown).

Linenscin OC2-induced ATP hydrolysis and inorganic phosphate efflux. Energized cells contain ca. 6.9 mM cytoplasmic...
ATP. Upon addition of linenscin OC2 (20 AU mg\(^{-1}\)) the intracellular ATP concentration decreased to a few percent of its original level (Fig. 4), but ATP was not found in the external medium. Concomitant with inducing ATP hydrolysis, linenscin OC2 induced a rapid efflux of inorganic phosphate, resulting in an increase of external Pi concentration from 2.8 to 5.9 mM in 10 min. Taking into account a cytoplasmic volume of 1 μl mg\(^{-1}\), this loss corresponds to a decrease in inorganic phosphate concentration of ca. 2.6 mM (Fig. 4B).

**Effect of temperature on the action of linenscin OC2.** Since *Listeria* is known as a psychrotrophic bacterium and linenscin OC2 acts on the cytoplasmic membrane, temperature effects on linenscin OC2 effectiveness were investigated. The action of linenscin OC2 (20 AU mg\(^{-1}\)) as a function of temperature was studied in *L. innocua* cells grown at 4, 13, and 37°C (Fig. 5). The initial rate of K\(^+\) efflux decreased significantly with decreasing assay temperatures regardless of the growth temperature. For a given assay temperature ranging from 18 to 37°C, the initial rate of K\(^+\) efflux decreased with decreasing growth temperatures. Freshly prepared *L. innocua* cells grown at 4, 13, and 37°C contained ca. 310, 460, and 600 nmol of K\(^+\) mg\(^{-1}\), respectively. So, initial rates of K\(^+\) efflux might be affected by the different cytoplasmic K\(^+\) concentrations. However, at 13°C no K\(^+\) efflux could be detected from cells grown at 37°C whereas significant K\(^+\) efflux was recorded from cells grown at 4 and 13°C (initial rate, ca. 20 nmol min\(^{-1}\) mg\(^{-1}\)).

**Influence of the proton motive force on linenscin OC2 activity.** In the presence of the protonophore CCCP (10 μM) the membrane potential of *L. innocua* cells was completely dissipated (data not shown). CCCP alone did not induce a significant K\(^+\) efflux from *L. innocua*. The addition of linenscin OC2 after preincubation with CCCP resulted in a rapid K\(^+\) efflux (785 nmol min\(^{-1}\) mg\(^{-1}\)) [Fig. 6]). Initial rates of K\(^+\) efflux were not significantly different in energized and deenergized cells, indicating that linenscin OC2 is active in the absence of a proton motive force. However, control cells lost all K\(^+\) whereas CCCP-treated cells maintained 40% of their cytoplasmic K\(^+\), suggesting that the efficiency of the permeabilization process could be affected in the absence of a proton motive force.

**FIG. 2.** Linenscin OC2-induced K\(^+\) efflux from *L. innocua*. Cells (0.49 mg ml\(^{-1}\)) were incubated at 37°C in the assay medium containing 0.6 mM KCl. (A) Linenscin OC2 (20 AU mg\(^{-1}\)) was added at time zero, and K\(^+\) efflux was determined as described in Materials and Methods. (B) The initial rate of K\(^+\) efflux as a function of linenscin OC2 dose is shown. The initial rates of K\(^+\) efflux were measured in the linear part of the different efflux curves.

**FIG. 3.** Effect of linenscin OC2 on *L. innocua* membrane potential. Cells (0.97 mg ml\(^{-1}\)) were incubated for 10 min at 37°C in the assay medium containing 10 μM [\(^{3}\)H]TPP\(^\circ\). (A) Linenscin OC2 (20 AU mg\(^{-1}\)) was added at time zero, and [\(^{3}\)H]TPP\(^\circ\) uptake was measured at various times. (B) Various doses of linenscin OC2 were added, and [\(^{3}\)H]TPP\(^\circ\) uptake was measured after 5 min of incubation. ΔΨ values were calculated, taking into account a cytoplasmic volume of 1 μl mg\(^{-1}\) and after correction for nonspecific binding, as described in Material and Methods.
**DISCUSSION**

The experiments described above show that the addition of low doses of linenscin OC2 (20 AU mg⁻¹) to *L. innocua* cells results in an immediate perturbation of the permeability properties of the cytoplasmic membrane and of the bacterial energetic state. Linenscin OC2 induces a loss of intracellular K⁺, depolarization of the cytoplasmic membrane, hydrolysis of internal ATP, efflux of inorganic phosphate, and stimulation and finally inhibition of the respiratory activity. These results demonstrate that the cytoplasmic membrane is the primary target for linenscin OC2.

Several observations indicate that linenscin OC2 does not disrupt the membrane structure by a detergent-like effect at low doses: (i) cell absorbance was not decreased when linenscin OC2 was added (data not shown); (ii) cell viability was not significantly changed by incubation of linenscin OC2 in the assay medium followed by dilution in TSBYE and plating on TSBYE agar plates; (iii) although we observed efflux of cytoplasmic K⁺ and inorganic phosphate, we could not detect any efflux of ATP. Thus, at low doses, the effects of linenscin OC2 might be due to the formation of pore-like structures in the cytoplasmic membrane. Linenscin OC2 causes an efflux of both cations (K⁺) and anions (inorganic phosphate), suggesting that these pores do not show ionic selectivity. The fact that no ATP

**FIG. 4.** Linenscin OC2-induced hydrolysis of intracellular ATP and efflux of inorganic phosphate. Cells (0.49 mg or 1.2 mg ml⁻¹; see Materials and Methods) were incubated at 37°C in the assay medium. (A) Cytoplasmic ATP in cells incubated for 5 min in the presence of increasing doses of linenscin OC2; (B) intracellular ATP level and extracellular inorganic phosphate in the presence of linenscin OC2 (20 AU mg⁻¹) (closed symbols) or in control cells (open symbols). Values of intracellular ATP are expressed as percentages of the initial concentration (100% corresponds to 6.9 mM). ATP and inorganic phosphate concentrations were determined as described in Materials and Methods.

**FIG. 5.** Effect of temperature on initial rate of linenscin OC2-induced K⁺ efflux in *L. innocua* cells grown at 4°C (◓), 13°C (北京时间), and 37°C (●). Cells (0.49 mg ml⁻¹) were incubated at various temperatures in the assay medium containing 0.6 mM KCl in the presence of linenscin OC2 (20 AU mg⁻¹). The initial rates of K⁺ efflux were measured as indicated in Materials and Methods and are given as the means of three determinations from cells grown at 13 and 37°C (error bars, standard deviations).

**FIG. 6.** Linenscin OC2-induced K⁺ efflux in the presence or absence of a proton motive force. Cells (0.49 mg ml⁻¹) were incubated at 37°C in the assay medium containing 0.6 mM KCl. Linenscin OC2 (20 AU mg⁻¹) (◇) or CCCP (10 μM) (●) was added (arrow 1). Linenscin OC2 was added 3 min after CCCP addition (arrow 2). K⁺ efflux was determined as described in Materials and Methods.
was found in the external medium suggests that these pore-like structures are not permeable to large molecules.

Linenscin OC2 inhibits the growth of gram-negative bacteria with an altered outer membrane permeability and of a large number of gram-positive bacteria (7). It is therefore likely that its activity is not receptor mediated. It is active on bacterial membranes and it is able to lyse cholesterol-containing erythrocyte membranes (7), suggesting that there is no requisite for a specific lipid composition of the cytoplasmic membrane.

Linenscin OC2 causes a loss of ca. 600 mM cytoplasmic potassium. This large efflux must be electrically compensated by a concomitant influx of cations or efflux of anions, which together cause membrane depolarization. The addition of linenscin OC2 caused an immediate hydrolysis of intracellular ATP and an efflux of inorganic phosphate. The intracellular ATP concentration decreased to a few percent of its original levels (0.49 mM compared to 6.9 mM), whereas no ATP was found externally and the efflux of inorganic phosphate corresponded to a decrease in cell concentration of ca. 2.6 mM.

Previously experiments have shown that membrane depolarization of L. monocytogenes upon treatment with a protonophore caused a slow decrease in the intracellular ATP concentration (ca. 20% in 10 min; see reference 2). Linenscin-induced membrane depolarization was apparently not responsible for ATP hydrolysis since it took place far more rapidly (ca. 75% in 2 min [Fig. 4B]) than the depletion expected from depolarization of cells. ATP hydrolysis might be partially caused by inorganic phosphate efflux and a subsequent shift in the ATP hydrolysis equilibrium as previously demonstrated in the case of colicin A, a pore-forming toxin active against Escherichia coli cells (19).

Linenscin OC2 stimulated the respiratory activity of L. innocua, suggesting that the rate of oxygen consumption of cells in the assay medium containing glucose is not maximal. Interestingly, addition of the protonophore CCCP in place of linenscin OC2 also caused a transient increase in oxygen consumption (data not shown). This suggests that L. innocua, unlike E. coli (10, 28), exhibits control of respiratory activity, i.e., this activity is regulated in such a manner that the rate of proton extrusion by the respiratory chain balances the rate of proton leak back across the cytoplasmic membrane. Stimulation of respiratory activity by linenscin OC2 and CCCP might be caused by membrane depolarization and proton entry. The linenscin OC2-induced increase of oxygen consumption was only transient. This is probably due to limitation in substrate availability as a consequence of the inhibition of glucose transport, which is proton motive force dependent (13).

The action of linenscin OC2 is severely reduced at low temperatures. The ordering of the lipid hydrocarbon chains, which takes place when the temperature is decreased, and consequently the decrease of membrane fluidity (18) are probably responsible for the decreased efficiency of linenscin OC2. These factors might prevent its insertion into the cytoplasmic membrane. Furthermore, if the pore-like structures are formed in the cytoplasmic membrane, then decreasing the temperature might prevent the lateral diffusion of individual linenscin OC2 molecules to form multimeric pores. Listeria adapts to growth at low temperatures by modifying membrane lipid composition (3, 26), thereby maintaining an optimum fluidity. Since ordering of the lipid hydrocarbon chains takes place at different temperatures depending on the lipid composition, this is probably why linenscin OC2 is active at 13°C only in cells grown at 4 and 13°C and not in cells grown at 37°C. Linenscin OC2 activity might also be limited by its low solubility at low temperatures: no K⁺ efflux was observed at 13°C from cells grown at 37°C at a dose of 20 AU mg⁻¹, whereas a K⁺ efflux could be recorded (initial rate, 50 nmol min⁻¹ mg⁻¹) from cells grown at 37°C at a dose of 100 AU mg⁻¹ (data not shown). These findings may have important implications for the use of B. linens OC2 as a cheese surface flora to prevent proliferation of L. monocytogenes during the ripening of red-smeared cheeses.

The induction of K⁺ efflux when linenscin OC2 was added to depolarized cells indicates that linenscin was active in the absence of a proton motive force. Initial rates of K⁺ efflux were not significantly different in energized and deenergized cells, but CCCP-treated cells did not lose all K⁺, suggesting that the efficiency of the permeabilization process could be affected in the absence of a proton motive force.

Effects and efficiency of linenscin OC2 on L. innocua in the assay medium used in this study and in TSBYE complete medium (7) can be compared. Incubation of cells in the assay medium containing low doses of linenscin OC2 (2.5 to 50 AU mg⁻¹) caused immediate perturbation of the membrane permeability and of the bacterial energetic state, although we did not observe any change in cell viability upon dilution in TSBYE and plating on TSBYE agar plates. Furthermore, in TSBYE medium containing low doses of linenscin OC2, we observed an increase of the cells' doubling time (25 to 130 AU mg⁻¹), bacteriostasis (130 to 850 AU mg⁻¹) and inhibition of peptidoglycan biosynthesis (850 AU mg⁻¹). It is likely that the formation of pore-like structures in the cytoplasmic membrane causes inhibition of L. innocua growth. These effects, observed at low doses of linenscin OC2, are nonlethal and reversible: a simple explanation might be that dilution of the cells in TSBYE medium induces dilution of the antibacterial substance and/or its preferential association with a component of this complete medium and, therefore, the disappearance of the pore-like structures. Thus, the membrane might recover its integrity and the cells might recover their physiological energetic state. At higher doses (>155 and ≤5,400 AU mg⁻¹), the effect of linenscin OC2 is bactericidal in the assay medium, although we did not observe any change in cell absorbance. Under these conditions, dilution of linenscin OC2 might not be enough for the membrane to recover its integrity. At very high doses, linenscin OC2 is bactericidal and bacteriolytic in the assay medium (>5,400 AU mg⁻¹) and in TSBYE medium (≥3,600 AU mg⁻¹). One possible explanation is that the formation of larger or more numerous pore-like structures could lead to cell lysis. Slow lysis, observed with high doses in TSBYE medium, could also be explained by a linenscin-induced degradation of peptidoglycan by autolysins in L. innocua (16) as suggested by the formation of protoplast-like cells in TSBYE medium (24).

In conclusion, linenscin OC2 might have a mode of action similar to that of bacteriocins like nisin, which, in addition to their cytoplasmic membrane-disruptive action, induce autolysis (5).

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REFERENCES


