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4 **ORIGINAL ARTICLE**

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6 **Pancreatic fistulae secondary to trypsinogen activation by *Pseudomonas aeruginosa* and**
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9 ***Enterobacter cloacae* infections after pancreatoduodenectomy**

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Abstract

Background Pancreatic fistula after pancreatoduodenectomy (PD) is associated with high mortality and morbidity. Trypsinogen activation and bacteria, although hypothesized to be inter-related etiopathogenetically, have not had their relationship and pathogenic mechanisms elucidated. This study investigated bacterial involvement in pancreatic juice activation perioperatively after PD at sites of pancreatic fistula formation.

Methods Eighty-two patients underwent PD; postoperative pancreatic fistulae were graded based on the International Study Group for Pancreatic Fistula grading criteria. Bacteria were isolated from cultures of drainage fluid. Digested peptides from trypsinogen and bacterial culture supernatants underwent SDS-PAGE separation and mass spectrometric analysis. Zymography was used to detect the trypsinogen activator.

Results *Pseudomonas aeruginosa* and *Enterobacter cloacae* isolated from drainage fluid in patients with grades B and C pancreatic fistulae could cause trypsinogen activation. Trypsinogen activation by *Pseudomonas aeruginosa* was preventable by the use of a serine protease inhibitor. A protease in the supernatant from *Pseudomonas aeruginosa*-positive cultures acted as the trypsinogen activator.

Conclusions Infection with *Pseudomonas aeruginosa* or *Enterobacter cloacae* perioperatively to PD entails secretion of a protease activator of trypsinogen to trypsin. Bacterial infection control in the perioperative PD period could be crucial to prevent development of pancreatic fistula.

Introduction

Pancreatoduodenectomy (PD) is associated with a high degree of surgical difficulty compared with other abdominal surgeries. It is imperative to understand the pathophysiology and involvement of physiologic and anatomic factors that follow digestive reconstruction in order to define adequate postoperative management after resection of the head of the pancreas. The incidences of complications and surgery-related deaths due to PD are higher, at 28–60% and 1–4%, respectively, when compared with other organ surgeries [1–4]. Early complications following PD include the formation of pancreatic and biliary fistulae, intraperitoneal bleeding, intra-abdominal abscesses, pulmonary complications, and delayed gastric emptying; diabetes, diarrhea, and malnutrition have been reported as late postoperative complications. Of these, pancreatic fistula, in particular, is a serious complication that could lead to surgery-related death, secondary to complications including intraperitoneal bleeding and intra-abdominal abscesses. The rate of incidence of pancreatic fistulae is reported to be 6–16% following PD [1–5].

Pancreatic juice—an isotonic, colorless, and transparent alkaline liquid secreted from the pancreas—contains digestive enzymes and a high concentration of bicarbonate ions (HCO_3^-). Enzymatic proteins constitute a large component of the pancreatic juice; trypsin, a crucial digestive enzyme that activates other digestive enzymes, accounts for 19% of the protein content [6]. Enterokinase activates trypsinogen to trypsin, which subsequently activates other proteolytic enzymes [7].

A pancreatic fistula after PD occurs due to autolysis caused by activated trypsin, resulting in tissue damage around the pancreatic ductal anastomosis. Patients with a pancreatic fistula develop intra-abdominal abscesses and intraperitoneal bleeding due to vascular rupture hemorrhage, often leading to sepsis and multiorgan failure. Therefore, it is very important to

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4 diagnose and treat pancreatic fistulae early during postoperative management. However, the
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6 mechanisms underlying the activation of pancreatic juice that has been attributed to invasive
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8 techniques, such as PD, remain unknown.
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11 A few studies have suggested a correlation between pancreatic fistulae and bacterial
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13 infection [5, 8]; however, the underlying pathogenesis remains to be clarified. In the present
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15 study, we examined the impact of bacterial infection on the activation of the enzymes in
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17 pancreatic juice perioperatively to PD by using strains isolated from the foci of infection at
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19 potential sites of pancreatic fistulae formation after PD.
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26 **Methods**

27 **Patients**

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29 From January 2009 to July 2014, 82 patients underwent PD at the Fukuoka University Hospital.
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31 Patients were assigned to two groups—a conventional perisurgical management and an intensive
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33 infection-control perisurgical management—based on their assessment periods. The intensive
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35 infection-control perioperative management included monitoring of preoperative cholangitis,
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37 antibiotic therapy based on cultures of preoperative bile duct discharge, repeated washing of the
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39 abdominal cavity during the operation, repeated change of surgical gloves during the operation,
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41 re-disinfection at the end of the surgery, and drain removal at an early stage. The conditions
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43 necessitating intervention through PD were invasive ductal cancer ($n = 34$), carcinoma of the
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45 ampulla of Vater ($n = 15$), bile duct cancer ($n = 6$), intraductal papillary mucinous neoplasms (n
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47 = 9), neuroendocrine tumors ($n = 1$), duodenal cancer ($n = 1$), and other types of cancer ($n = 16$).
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49 Of the patients who underwent PD, 47 were men and 35 were women (age: range, 41–82 years;
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51 mean [SD], 68.63 [8.55] years). In 28 patients, preoperative biliary drainage (percutaneous
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53 transhepatic biliary drainage in eight patients, endoscopic nasobiliary drainage in one patient,
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4 and endoscopic biliary tube stenting in 19 patients) was performed for the treatment of
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6 obstructive jaundice or cholangitis. Preoperative cholangitis occurred in 11 patients and was
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8 surgically resolved in all cases. The present study was conducted in accordance with the
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10 principles of the Declaration of Helsinki. Sample collection and administrative procedures in this
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12 study were conducted in accordance with the code of ethics specified by the Institutional Ethics
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14 Committee of the Fukuoka University Hospital.
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21 Operative technique

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23 For all patients, as the first step in reconstruction, the proximal jejunum was mobilized laterally
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25 through the right side of the transverse mesocolon via the retrocolic route. Next, a duct-to-
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27 mucosa anastomosis was performed between the pancreatic duct and jejunal mucosa and secured
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29 with eight interrupted 5-0 PDS-II sutures (polydioxanone; Johnson and Johnson Co.), regardless
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31 of the size of the pancreatic duct. A 7.5-French polyethylene pancreatic duct surgical drainage
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33 tube (Sumitomo Bakelite Co., Japan) was placed in the pancreatic duct and exteriorized through
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35 the jejunal limb. Thereafter, an anastomosis was performed between the remnant pancreatic
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37 capsule, the parenchyma, and the jejunal seromuscular layer with the use of interrupted 3-0 nylon
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39 sutures (Alfresa Pharma, Co., Japan). The pancreatic duct tube was removed on postoperative
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41 day (POD) 21. A one-layer end-to-side hepatojejunostomy was performed using 4-0 PDS-II
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43 sutures (Johnson and Johnson Co.), placed 5 cm distal to the site of pancreatojejunostomy. A
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45 retrocolic end-to-side duodenojejunostomy was performed 40 cm distal to the site of the
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47 hepatojejunostomy. A double drain was placed within the dorsal end of the bile duct–jejunum
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49 anastomosis and the underside of the caudate lobe of the liver, and an 8-mm Penrose, silicon,
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51 multitubular flat drain was placed in the dorsal end of the pancreatojejunostomy. The drain was
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4 removed on confirming clear discharge, with no leakage of pancreatic juice or bacterial
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6 contamination.
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10 11 Pancreatic fistula evaluation

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14 The presence of a pancreatic fistula was defined by the appearance of an amylase-rich
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16 measurable drainage fluid from an intraoperatively placed drain. The presence of a postoperative
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18 pancreatic fistula was defined as the detection of amylase level ≥ 3 times the normal serum
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20 amylase level in the drainage fluid on POD 3. The following three grades of fistula severity were
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22 assessed according to the International Study Group for Pancreatic Fistula (ISGPF) grading
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24 criteria: Grade A, “transient fistula,” no clinical impact; Grade B, requiring a change in
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26 management or adjustment of the clinical pathway; and Grade C, requiring a major change in
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28 clinical management or deviation from the normal clinical pathway [9].
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36 Drainage management and drainage fluid culture

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38 The characteristics and amount of drainage, amylase level in the drainage, blood test parameters,
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40 and clinical symptoms were used as references for drainage management. The amylase level in
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42 the drained fluid was measured, and pure cultures from the drainage fluid specimens obtained
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44 from all patients on PODs 1, 3, 5, and 7 were obtained. We investigated the samples for bacteria.
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46 The drain was removed when there were no signs of bacterial infection and the amylase level in
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48 the drained fluid was ≤ 3 times that in the serum. In patients showing signs of infection, the drain
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50 was replaced under fluoroscopic guidance at a suitable time. Furthermore, in patients showing
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52 fluid accumulation on computed tomography (CT), additional surgical drainage was performed
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54 under ultrasonographic guidance.
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Bacterial strains and growth conditions

Pseudomonas aeruginosa was isolated from the drained fluid of a patient diagnosed with an ISGPF Grade B pancreatic fistula. *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, *Enterobacter cloacae* complex, and *Enterococcus faecalis* were isolated from patients with intra-abdominal abscess formation, urinary tract infection, or enteritis. A *Pseudomonas aeruginosa* standard strain (ATCC 9027) was purchased from Microbiologics (St. Cloud, Minnesota, USA). Ten strains of *Pseudomonas aeruginosa*; two strains of *Escherichia coli*; and a single strain each of MRSA, *Enterobacter cloacae* complex, and *Enterococcus faecalis* were used. Each bacterium was grown in liquid Tryptone Soya Broth (TSB) medium or Brain Heart Infusion (BHI) medium at 37°C with agitation until the culture reached an optical density 600 (OD₆₀₀) value of 1.0. When required, the bacterial culture was centrifuged (10,000 × g for 3 minutes), and the supernatant was filtered using a Millex Syringe-driven 0.22-µm filter unit (Millipore, Billerica, Massachusetts, USA).

Trypsinogen activation assay and SDS–PAGE

Commercial trypsinogen was obtained from Sigma (bovine pancreatic trypsinogen T1143). Trypsinogen was dissolved with phosphate-buffered saline (PBS) or acetate buffer (pH 3.6). Fetal bovine serum (FBS) was used as the control. Next, 5 µg of trypsinogen was mixed with 5 µL of filtered bacterial culture supernatant, and the mixture was incubated at 37°C for 0, 3, 6, 9, 12, and 24 hours. Each of trypsinogen, FBS, and trypsinogen–FBS mixture was used as a control. Samples were mixed with 2× sample buffer and heated at 95°C for 5 minutes. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using 12% (w/v)

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4 polyacrylamide gel according to the method described by Laemmli [10]. After electrophoresis,
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6 the gel was stained using the Rapid Stain CBB kit (NACALAI TESQUE, Kyoto, Japan) to
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8 visualize the proteins. When required, phenylmethylsulfonyl fluoride (PMSF) or pepstatin A
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10 (Pep) was added to the assay solution at concentrations of 1 mmol/L and 1 μ mol/L, respectively.
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15 16 Mass spectrometric analysis

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18 The samples were separated via SDS-PAGE and stained with Coomassie Brilliant Blue,
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20 followed by in-gel digestion [11] with 10 μ g/mL of porcine trypsin (Promega) or 17 μ g/mL of
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22 chymotrypsin (Roche Diagnostics). Trypsin and chymotrypsin were incubated at 37°C and 25°C,
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24 respectively. The digested peptides were eluted with 0.1% formic acid and subjected to
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26 matrix-assisted laser desorption/ionization using time-of-flight mass spectrometry (MALDI-TOF
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28 MS) analysis or liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.
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30 MALDI-TOF MS was performed on an AXIMA-TOF² instrument (Shimadzu) in the positive
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32 reflection mode. In order to identify the amino acid sequences of the major peptides, an
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34 LC-MS/MS analysis was performed on an LCMS-IT-TOF instrument (Shimadzu) interfaced
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36 with a nano reverse-phase liquid chromatography system (Shimadzu) [12]. The MS/MS data
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38 were acquired in the datum-dependent mode using the LCMS solution software program
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40 (Shimadzu) and converted to a single text file (containing the observed precursor peptide m/z ,
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42 fragment ion m/z , and intensity values) with Mascot Distiller (Matrix Science). The MS/MS data
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44 were analyzed using the Mascot software program (Matrix Science). The search parameters were
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46 as follows: database, bovine trypsinogen (246 amino acid residues; **Table 1**); enzyme, all; and
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48 variable modification, carbamidomethyl (Cys), propionamide (Cys), and oxidation (Met).
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Trypsinogen zymography

For zymography, we modified a previously reported method [13]. In brief, 12% polyacrylamide gel containing 0.1% trypsinogen was employed to detect the trypsinogen activator. After electrophoresis, the gel was rinsed in 2.5% Triton X-100 for 40 minutes and an enzymatic reaction was employed in 50 mmol/L Tris-HCl (pH 8.8) at 37°C for 6 hours. Thereafter, the gel was stained using the Rapid Stain CBB kit.

Results

Incidence and etiopathogeny of pancreatic fistulae in the conventional perisurgical management group

Initially, bacteria isolated from the cultures of drainage fluid from patients in the conventional perisurgical management group from January 2009 to May 2013 were investigated. The bacterial species isolated included *Enterococcus faecalis* ($n = 12$, 24.0%), *Enterobacter cloacae* ($n = 5$, 10.0%), *Pseudomonas aeruginosa* ($n = 4$, 8.0%), and others including *Acinetobacter baumannii*, *Citrobacter freundii*, and *Klebsiella pneumoniae*. The incidence of Grade B/C pancreatic fistulae was 18.0%. The causative agents of Grade B/C pancreatic fistula formation were *Enterococcus faecalis* ($n = 6$, 66.7%), *Enterobacter cloacae* ($n = 3$, 33.3%), and *Pseudomonas aeruginosa* ($n = 4$, 44.4%). *Enterococcus faecalis* and *Enterobacter cloacae* were found in the cultures of six (14.6%) and two (4.9%) patients, respectively, with non-pancreatic and Grade A pancreatic fistulae; however, *Pseudomonas aeruginosa* was found only in patients with Grade B/C pancreatic fistulae (**Table 2**). These results suggest that infection with *Pseudomonas aeruginosa* is strongly related to the incidence of Grade B/C pancreatic fistulae. In consideration of this result, we carried out our next experiment.

Activation of trypsinogen by *Pseudomonas aeruginosa*

Processing of trypsinogen and the subsequent trypsin activation is one of the key factors in severe (Grade B/C) pancreatic fistulae. Our survey clearly showed an association between *Pseudomonas aeruginosa* infection and formation of severe pancreatic fistulae (**Table 2**). *Pseudomonas aeruginosa* is a common pathogen isolated from patients and is a cause of opportunistic infections. In addition, this bacterial species displays a protein secretion mechanism, called the Type III secretion system (T3SS), through which it secretes various effector proteins, including proteases and toxins, to modify the external environment [14]. Therefore, we investigated whether *Pseudomonas aeruginosa* secretes any proteins capable of activating trypsinogen. When purified bovine trypsinogen was incubated with the culture supernatant of *Pseudomonas aeruginosa* (a clinical isolated strain from patients with pancreatic fistulae), a 24-kDa band indicating a time-dependent decrease in trypsinogen, and two small bands corresponding to 13 and 10 kDa at 3 hours after incubation was observed. In contrast, when trypsinogen was incubated with the bacterial culture of *Pseudomonas aeruginosa*, a smaller 24-kDa trypsinogen band indicating a lesser decrease were observed, and the small bands did not appear until 24 hours after incubation. The trypsinogen band did not diminish for the trypsinogen–FBS mixture until 24 hours after incubation, and the FBS-only samples did not yield a 24-kDa band (**Fig. 1a**). These results suggest that the supernatant of bacterial culture is related to a greater decrease in trypsinogen and produces two small fragments when compared with the *Pseudomonas aeruginosa* bacterial component.

Pseudomonas aeruginosa and *Enterobacter cloacae* induce activation of trypsinogen

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4 Besides *Pseudomonas aeruginosa*, several bacteria such as *Enterococcus faecalis* and
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6 *Enterobacter cloacae* were isolated from patients with Grade B/C pancreatic fistulae after PD
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8 (Table 1). Therefore, it was important to assess if these bacteria also have the ability to induce
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10 trypsinogen activation similar to *Pseudomonas aeruginosa*. Therefore, we examined a
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12 trypsinogen activation assay using nine strains of *Pseudomonas aeruginosa* clinical isolates and
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14 a reference strain, MRSA clinical isolates, *Escherichia coli*, *Enterobacter cloacae* complex, and
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16 *Enterococcus faecalis*. Trypsinogen was dissolved in acetate buffer and incubated with the
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18 supernatant from the bacterial culture for 12 hours, and trypsinogen processing was assessed via
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20 SDS–PAGE. When trypsinogen was incubated with the TSB or BHI medium, the 24-kDa
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22 trypsinogen band did not decrease even at 12 hours after incubation. In contrast, nine of the ten
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24 *Pseudomonas aeruginosa* bacterial culture supernatants showed a trypsinogen reduction activity,
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26 except for the ‘*Pseudomonas aeruginosa* 1’ clinical isolate. Reduction in trypsinogen was also
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28 observed for the *Enterobacter cloacae* complex-cultured supernatant, whereas MRSA,
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30 *Escherichia coli*, and *Enterococcus faecalis* culture supernatants did not show any decrease (Figs
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32 **1b and 1c**). These results clearly suggest that the *Pseudomonas aeruginosa* and *Enterobacter*
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34 *cloacae* complex, but not MRSA, *Escherichia coli*, or *Enterococcus faecalis*, secrete a
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36 trypsinogen activator into the culture supernatants during their growth periods.
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48 Identification of trypsin using mass spectrometry

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50 To determine both the small 10- and 13-kDa bands that were observed in the sample in which
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52 trypsinogen was incubated with *Pseudomonas aeruginosa*-cultured supernatant, we performed a
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54 MALDI-TOF MS analysis. The complete amino acid sequence of trypsinogen is shown in Table
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56 **1**. The 10- and 13-kDa bands shown in Fig. 1a were removed, and mass spectrometry was
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4 conducted. Using MALDI-TOF-MS, the mass spectral peak of bovine trypsinogen was detected
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6 in agreement with the theory, and the amino acid at the peak was examined using an MS/MS
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8 analysis with nano-LC coupled to LCMS-IT-TOF. To determine which part of the amino acid
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10 sequence was equivalent to the band detected on SDS-PAGE, a complete amino acid sequence
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12 of bovine trypsinogen was examined. Mass spectrometry was conducted after the 13-kDa band
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14 was degraded with chymotrypsin and porcine trypsin, and the respective mass spectral peak was
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16 identified. The peptide detected using this procedure is shown in **Supplementary Fig. 1a**, and
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18 was thought to be the α -trypsin chain-1. Mass spectrometry of the 10-kDa band showed a mass
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20 spectral peak, with the detected peptide shown in **Supplementary Fig. 1b**, which led to the
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22 conclusion that it was the α -trypsin chain-2.
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29 Therefore, the results showed that the culture supernatant of *Pseudomonas aeruginosa*
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31 activated trypsinogen into trypsin.
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36 Prevention of trypsinogen activation by *Pseudomonas aeruginosa* and *Enterobacter cloacae*
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38 using a serine protease inhibitor
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41 In the normal environment, pancreatic trypsinogen is typically activated by enterokinase, which
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43 is the serine protease produced by duodenal mucosa [7], by cleaving the N-terminal activation
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45 peptide of trypsinogen followed by autocatalytic activation [15]. Cleavage of trypsinogen by
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47 serine protease is the trigger for activation; therefore, it is possible that the trypsinogen-cleaving
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49 factor secreted by *Pseudomonas aeruginosa* and *Enterobacter cloacae* is one of the serine
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51 proteases. A serine protease inhibitor PMSF and an aspartic protease inhibitor pepstatin A were
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53 applied to the trypsinogen activation assay. In the presence of 1 mmol/L PMSF, the trypsinogen
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55 band mostly persisted at 12 hours after incubation, whereas it did not persist following
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4 incubation with 1 $\mu\text{mol/L}$ pepstatin A, indicating that pepstatin A did not inhibit trypsinogen
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6 processing and confirming that trypsinogen activation was inhibited by only the serine protease
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8 inhibitor (**Fig. 2**). This result suggests the possibility that the trypsinogen-activating factor
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10 secreted from *Pseudomonas aeruginosa* and *Enterobacter cloacae* possesses serine protease
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12 activity and subsequently activates trypsinogen.
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18 19 Zymography

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21 To detect both the number and the size of trypsinogen-activating factor secreted from
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23 *Pseudomonas aeruginosa*, we conducted zymography for trypsinogen that contains unprocessed
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25 trypsinogen in the polyacrylamide gel for the trypsinogen-activating protease in the
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27 *Pseudomonas aeruginosa* culture supernatant. We found a single ~50-kDa band in the
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29 supernatant (**Fig. 3**). However, this band was not detected for *Enterobacter cloacae* under the
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31 same conditions.
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36 These results suggest that the protease in the supernatant of *Pseudomonas aeruginosa*
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38 acts as a trypsinogen activator.
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42 43 Incidence and etiopathogeny of pancreatic fistulae in the intensive infection-control perisurgical 44 45 management group

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47 Intensive infection control during perisurgical management was carried out from April 2013. In
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49 the intensive infection-control perisurgical management group, the bacteria isolated from the
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51 cultures of drainage fluid included *Enterococcus faecalis* ($n = 7$, 21.9%), *Enterobacter cloacae*
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53 ($n = 4$, 12.5%), and *Pseudomonas aeruginosa* ($n = 1$, 3.1%). The detection rate of bacteria
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55 isolated from the drained fluid of patients who underwent PD was reduced by intensive
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4 infection-control perisurgical management. The incidence of Grade B/C pancreatic fistulae was
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6 6.3%, and had reduced dramatically when compared with the conventional perisurgical
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8 management group. The causative agents of Grade B/C pancreatic fistula formation were
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10 *Enterococcus faecalis* ($n = 1$, 50.0%), *Enterobacter cloacae* ($n = 2$, 100.0%), and *Pseudomonas*
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12 *aeruginosa* ($n = 1$, 50.0%). *Enterococcus faecalis* and *Enterobacter cloacae* were found in the
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14 cultures of six (20.0%) and two (6.6%) patients, respectively, with non-pancreatic and Grade A
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16 pancreatic fistulae; however, *Pseudomonas aeruginosa* was found only in patients with Grade
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18 B/C pancreatic fistulae (**Table 3**).

26 Association of bacterial strain with grade of pancreatic fistulae

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28 We investigated the association between bacterial strain and Grade B/C pancreatic fistulae in all
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30 patients. All *Pseudomonas aeruginosa* and more than half of *Enterobacter cloacae* infections
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32 were detected in Grade B/C pancreatic fistulae. However, only one of seven cases (14.3%) with
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34 *Enterococcus faecalis* infection was reported to have developed Grade B/C pancreatic fistulae in
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36 the intensive infection-control perisurgical management group (**Fig. 4**). This result does not
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38 contradict our laboratory findings.
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45 **Discussion**

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47 PD is a highly invasive operative technique used for treating cancer of the pancreatic head and is
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49 known for its high incidence of complications. In particular, complications of pancreatic fistulae
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51 cause circumferential tissue damage due to the autodestructive effects of activated pancreatic
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53 juice, which could subsequently result in intra-abdominal abscess development, intraperitoneal
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55 hemorrhage due to vessel rupture, sepsis, multiorgan failure, and even death. Therefore, various
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4 studies have been conducted to understand the mechanisms for the prevention of pancreatic
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6 fistulae.
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9 With regard to risk factors for pancreatic fistulae, randomized clinical trials evaluating
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11 pancreatic fistula formation have reported that the presence of soft parenchyma [16], a
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13 nondilated pancreatic duct [17, 18], and technical factors such as non-pancreatic duct stent
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15 placement [19] are closely related. Several studies have investigated measures to prevent
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17 pancreatic fistula by assessing the comparative efficacy of: (1) pancreas–intestine and pancreas–
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19 stomach anastomoses as a reconstruction technique [20–22]; (2) the administration of medical
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21 agents known to have suppressive effects on pancreatic exocrine secretion [23–26]; and (3) the
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23 application of fibrin glue to sites of pancreas–stomach anastomosis [27, 28]. None of these
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25 studies have shown any decrease in the incidence of pancreatic fistulae, and there is still no
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27 consensus on this topic.
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33 The activation of trypsinogen is an extremely important element in the development of
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35 pancreatitis [29–32]. Protease, a strong pancreatic enzyme, is first secreted in the form of
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37 inactive zymogen (proenzyme). After pancreatic juice enters the duodenum, trypsinogen is
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39 activated by enterokinase on the mucosal epithelium and becomes trypsin [7]. Trypsin then
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41 activates chymotrypsinogen, other zymogens, and even itself. In brief, once trypsin is generated,
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43 its activation further progresses via a self-catalytic reaction. Trypsin, the activated form of
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45 trypsinogen, is crucial as the key enzyme in the pancreas and plays central roles in the cascade of
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47 activation of various enzymes, inhibitory feedback of pancreatic exocrine secretion, the
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49 development and pathology of pancreatitis, and so on [6]. Therefore, to examine the
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51 development of pancreatic fistulae after PD, it is important to first consider the pathophysiology
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53 specific to the formation of pancreatic fistulae, such as the involvement of physiologic and
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4 anatomic factors after digestive reconstruction, stress caused by the operative procedure, and
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6 perioperative infectious diseases, as well as investigate factors that activate trypsinogen and
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8 consequent lead to the development of pancreatic fistulae.
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11 In addition, a few researchers have reported a correlation between pancreatic fistulae
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13 and bacterial infection, indicating the possibility of involvement of bacterial infection in the
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15 activation of pancreatic juice [5, 8]. In recent years, many reports have stated that long-term
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17 placement of drainage after PD is a risk factor for increased incidence of postoperative
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19 complications, such as pancreatic fistula and progression toward higher grades of such fistulae,
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21 and that drains should be removed at an early stage [33–35]. Drains placed surgically increase
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23 the risk of intra-abdominal infections by providing a route for ascending infections.
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29 In the present study, we examined the impact of bacterial infection on the activation of
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31 pancreatic juice in the perioperative PD period by using strains isolated from the foci of infection
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33 in patients who had developed pancreatic fistula after PD. Initially, examination of bacteria
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35 isolated from the drainage fluid of patients treated with PD at our hospital showed that
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37 *Pseudomonas aeruginosa*, *Enterobacter cloacae*, and *Enterococcus faecalis* accounted for most
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39 of the bacteria found in Grade B/C pancreatic fistulae. However, *Pseudomonas aeruginosa* was
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41 not found in patients with non-pancreatic and Grade A pancreatic fistulae, although it was
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43 common in those with Grade B/C pancreatic fistulae. These findings suggest that bacterial
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45 infection caused by *Pseudomonas aeruginosa* is involved in the development of higher-grade
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47 pancreatic fistulae. Therefore, we analyzed the mechanism by which *Pseudomonas aeruginosa*
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49 acts as an activator of trypsinogen. *Pseudomonas aeruginosa* is an obligate aerobe and
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51 non-fermentative Gram-negative bacillus. It is an important pathogen causing infectious diseases
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53 in hosts with immunodeficiency, the so-called opportunistic infections. Postoperative
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4 *Pseudomonas aeruginosa* infection is difficult to treat and tends to be prolonged, sometimes
5
6 leading to life-threatening complications. This bacterial species directly infuses toxins and
7
8 effectors into host cells via the type III secretion system to damage the cells. These systems play
9
10 a critical role in the resistance of *Pseudomonas aeruginosa* to macrophages. In addition, this
11
12 bacterium produces various proteolytic enzymes, including elastase, LasA, *Pseudomonas*
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14 *aeruginosa* alkaline protease, and Ps-1 protease, as etiopathogenic factors [13, 36–38].
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19 In the present study, the isolated and cultivated supernatants of *Pseudomonas*
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21 *aeruginosa* and trypsinogen were initially cocultured. The disappearance of the 24-kDa band of
22
23 trypsinogen and the appearance of new bands near 13 and 10 kDa were subsequently confirmed
24
25 with SDS–PAGE. Furthermore, mass spectrometry revealed that the 13- and 10-kDa bands
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27 corresponded to α -trypsin chain-1 and α -trypsin chain-2, respectively. In addition, this
28
29 phenomenon was controlled by a serine protease inhibitor. The results suggest that a kind of
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31 protease in the culture supernatant of *Pseudomonas aeruginosa* induced the activation of
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33 trypsinogen into trypsin.
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38 Next, we performed trypsinogen zymography to identify the protease-activating
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40 trypsinogen, and the results thus obtained suggested the involvement of a protease equivalent to
41
42 the 50-kDa band. Previous studies have also reported that casein zymography detects alkaline
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44 protease as a band near 50 kDa [13, 39].
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48 Similarly, the culture supernatant of *Enterobacter cloacae* induced the activation of
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50 trypsinogen, which was inhibited by a serine protease inhibitor, similar to that of *Pseudomonas*
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52 *aeruginosa*-induced activation. *Enterobacter cloacae* are clearly opportunistic pathogens and
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54 rarely cause disease in otherwise healthy individuals. As opportunistic pathogens have only
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56 recently gained importance as causes of nosocomial infections, very little is known about the
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4 factors affecting their pathogenicity and virulence [40]. *Enterobacter cloacae* infection was
5 commonly seen with Grade B/C pancreatic fistulae in this study.
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9 This study verified that the protease in the culture supernatants of *Pseudomonas*
10 *aeruginosa* and *Enterobacter cloacae*, which are isolated from the foci of infection in patients
11 who developed pancreatic fistula after PD, activates trypsinogen into trypsin. This study is the
12 first report to establish an association between trypsinogen activation and bacterial infection.
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19 Based on our investigation of the incidence of pancreatic fistulae and causative agents
20 treated with conventional perisurgical management, we believed that infection control in the
21 perioperative PD period is a very important aspect of care. Therefore, we developed and
22 introduced an intensive infection-control perisurgical management. The detection rate of bacteria
23 isolated from the drained fluid of patients who underwent PD was reduced by intensive infection
24 control. In the intensive infection-control perisurgical management group, the incidence of Grade
25 B/C pancreatic fistulae was 6.3%, much lower than the 18.0% seen in the conventional
26 perisurgical management group. It was suspected that *Pseudomonas aeruginosa* and
27 *Enterobacter cloacae* were related to Grade B/C pancreatic fistulae. However, despite intensive
28 infection control during perisurgical management, we could not prevent Grade B/C pancreatic
29 fistulae. The surgical technique and patient risk factors (soft parenchyma or nondilated
30 pancreatic duct), we believe, need to be considered as contributory factors. Furthermore, strict
31 infection-control measures should be instituted for the prevention of *Pseudomonas aeruginosa*
32 and *Enterobacter cloacae* infections. In the present study, cultures of intraoperative duodenal
33 juice and bile for intensive infection control were examined during the perisurgical period.
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4 patients with Grade B/C pancreatic fistulae in the intensive infection-control perisurgical
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6 management group. Therefore, we believe that cultures of intraoperative duodenal juice and bile
7
8 could be considered as predictors of Grade B/C pancreatic fistulae risk.
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11 In conclusion, infection with *Pseudomonas aeruginosa* and *Enterobacter cloacae* during
12
13 the perioperative PD period activates pancreatic juice and induces the progression of pancreatic
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15 fistulae into higher-grade fistulae. Therefore, providing measures of bacterial infection control,
16
17 such as cholangitis treatment, drainage management, monitoring cultures of intraoperative
18
19 duodenal juice and bile, and antibiotic therapy, in the perioperative PD period is crucial to
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21 prevent the development of pancreatic fistulae.
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4 **Author Contributions**
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9 **Study design:** Kanefumi Yamashita, Takamitsu Sasaki, Ryota Itoh, Kenji Hiromatsu, Yuichi
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28 Yuichi Yamashita
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30 **Statistical advice:** Not applicable
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11 **Conflict of interests**
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14 None to be declared.
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59 **Figure legends**
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4 **Fig. 1** Incubation of culture supernatant of various bacteria with trypsinogen. **(a)** Recombinant
5 trypsinogen (TNG) was incubated with bacterial cells or culture supernatants of *Pseudomonas*
6 *aeruginosa* at 37°C. Samples were harvested at indicated time points and applied onto SDS–
7 PAGE. Fetal bovine serum (FBS) was used as a control. **(b)** Incubation of culture supernatant of
8 *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*,
9 *Enterobacter cloacae* complex, and trypsinogen. Nine clinical isolates and one reference strain
10 of *Pseudomonas aeruginosa*, two *Escherichia coli* clinical isolates, MRSA, and *Enterobacter*
11 *cloacae* complex were separately incubated with trypsinogen as described in **Fig. 1a**. As for the
12 control, trypsinogen was also incubated with the TSB culture medium. **(c)** Incubation of culture
13 supernatant of *Pseudomonas aeruginosa*, *Enterobacter cloacae* complex, *Enterococcus faecalis*,
14 with trypsinogen. Each culture supernatant of *Pseudomonas aeruginosa*, *Enterobacter cloacae*
15 complex, and *Enterococcus faecalis* was incubated with recombinant trypsinogen as shown in
16 **Fig. 1a**. As for the control, trypsinogen was also incubated with BHI culture medium.

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19 **Fig. 2** Trypsinogen activation by the culture supernatants of *Pseudomonas aeruginosa* and
20 *Enterobacter cloacae* complex were inhibited by the protease inhibitor. Trypsinogen was
21 separately incubated with the supernatants of *Pseudomonas aeruginosa* or *Enterobacter cloacae*
22 complex cultures in the presence or absence of 1 mmol/L of serine protease inhibitor
23 phenylmethylsulfonyl fluoride (PMSF) or 1 µmol/L of aspartic protease inhibitor pepstatin A
24 (Pep) for 24 hours. Each sample was analyzed by SDS–PAGE and Coomassie blue staining.

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27 **Fig. 3** Trypsinogen zymography. Each TSB medium or filter-concentrated *Pseudomonas*
28 *aeruginosa* culture supernatant was loaded onto trypsinogen-containing SDS–PAGE. After
29 electrophoresis, the gel was washed and incubated at 37°C for 6 hours. Trypsinogen degradation
30 was detected via Coomassie blue staining.

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4 **Fig. 4** Association of bacterial strains with grade of pancreatic fistulae. The association of
5 bacterial strains isolated from the drainage fluid of patients who underwent PD with
6 conventional infection-control perisurgical management (Con) or intensive infection-control
7 perisurgical management (Int) and grade of pancreatic fistulae is shown.
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16 **Supplementary Fig. 1** Analysis of trypsinogen-decomposed products using mass spectrometry.

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18 Mass spectrometry was conducted using an MS/MS analysis following the decomposition of the
19 13- and 10-kDa bands (**Fig. 1a**), as detected on SDS-PAGE with chymotrypsin and porcine
20 trypsin. This figure shows the amino acids detected based on the mass spectral peak.
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28 **List of tables**

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31 **Table 1** The complete amino acid sequence of trypsinogen (bovine)
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33 **Table 2** Isolated bacteria in the drained discharge fluid following pancreatoduodenectomy with
34 conventional perisurgical management
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38 **Table 3** Isolated bacteria in the drained discharge fluid following pancreatoduodenectomy with
39 intensive infection-control perisurgical management
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Table 1. The complete amino acid sequence of trypsinogen (bovine)

1	MKTFIFLALL	GAAVAFPVDD	DDKIVGGYTC	GANTVPYQVS	LNSGYHFCGG
51	SLINSQWVVS	AAHCYKSGIQ	VRLGEDNINV	VEGNEQFISA	SKSIVHPSYN
101	SNTLNNDIML	IKLKSAASLN	SRVASISLPT	SCASAGTQCL	ISGWGNTKSS
151	GTSYPDVLKC	LKAPILSDSS	CKSAYPGQIT	SNMFCAGYLE	GGKDSCQGDS
201	GGPVVCSGKL	QGIVSWGSGC	AQKNKPGVYT	KVCNYVSWIK	QTIASN

Table 2. Isolated bacteria in the closed-drain discharge fluid following pancreatoduodenectomy with conventional perisurgical management

	Non-PF/Grade A <i>n</i> = 41	Grade B/C <i>n</i> = 9 (18.0%) ^a	All <i>n</i> = 50
	<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)
<i>Enterococcus faecalis</i>	6 (14.6)	6 (66.7)	12 (24.0)
<i>Enterobacter cloacae</i>	2 (4.9)	3 (33.3)	5 (10.0)
<i>Pseudomonas aeruginosa</i>	0 (0.0)	4 (44.4)	4 (8.0)
<i>Escherichia coli</i>	0 (0.0)	2 (22.2)	2 (4.0)
Others	1 (2.4)	2 (22.2)	3 (6.0)
Total	7 (17.1)	9 (100.0)	16 (32.0)

Non-PF, non-pancreatic fistula

^a Incidence of Grade B/C pancreatic fistula. Infection with a single organism, 8 cases; infection with two organisms, 6 cases; infection with three organisms, 2 cases

Table 3. Isolated bacteria in the closed-drain discharge fluid following pancreatoduodenectomy with intensive infection control perisurgical management

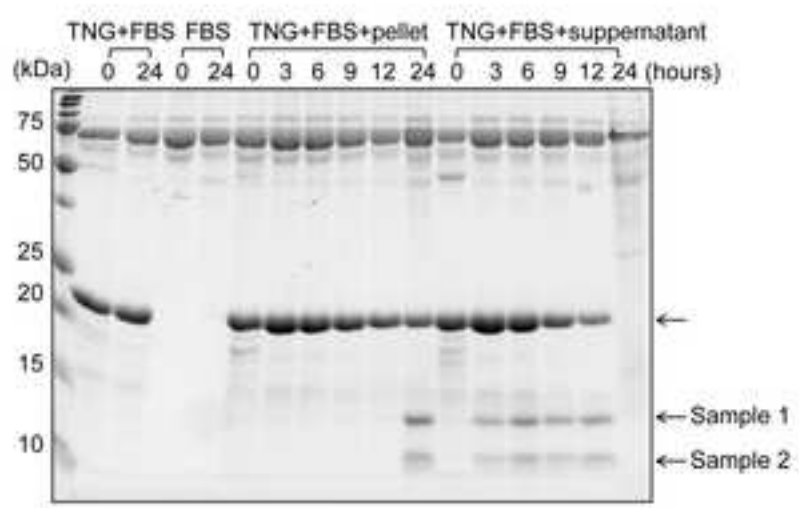
	Non-PF/Grade A <i>n</i> = 30	Grade B/C <i>n</i> = 2 (6.3%) ^a	All <i>n</i> = 32
	<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)
<i>Enterococcus faecalis</i>	6 (20.0)	1 (50.0)	7 (21.9)
<i>Enterobacter cloacae</i>	2 (6.6)	2 (100.0)	4 (12.5)
<i>Pseudomonas aeruginosa</i>	0 (0.0)	1 (50.0)	1 (3.1)
<i>Escherichia coli</i>	1 (3.3)	0 (0.0)	1 (3.1)
Others	1 (3.3)	0 (0.0)	1 (3.1)
Total	7 (23.3)	2 (100.0)	9 (28.1)

Non-PF, non-pancreatic fistula

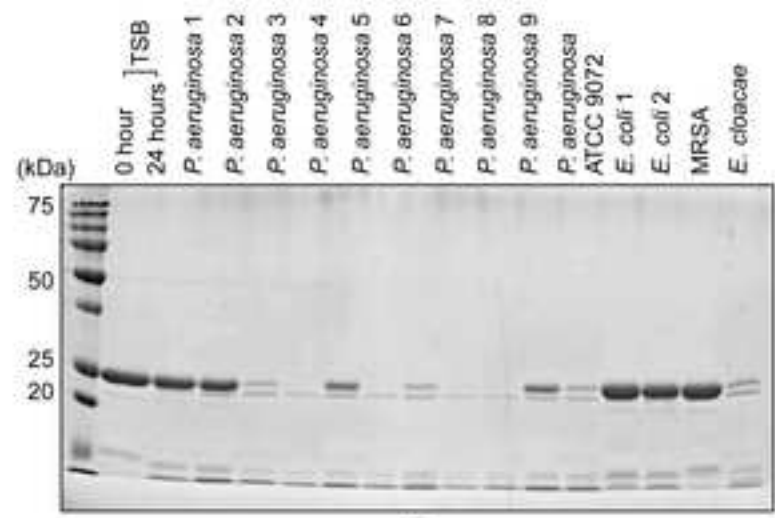
^a Incidence of Grade B/C pancreatic fistula

Infection with a single organism, 5 cases; infection with two organisms, 3 cases; infection with three organisms, 1 case.

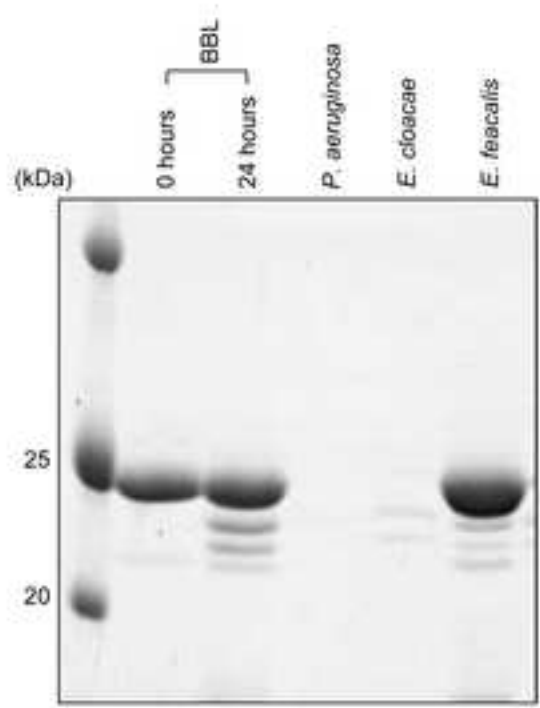
Figure 1
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Figure 2

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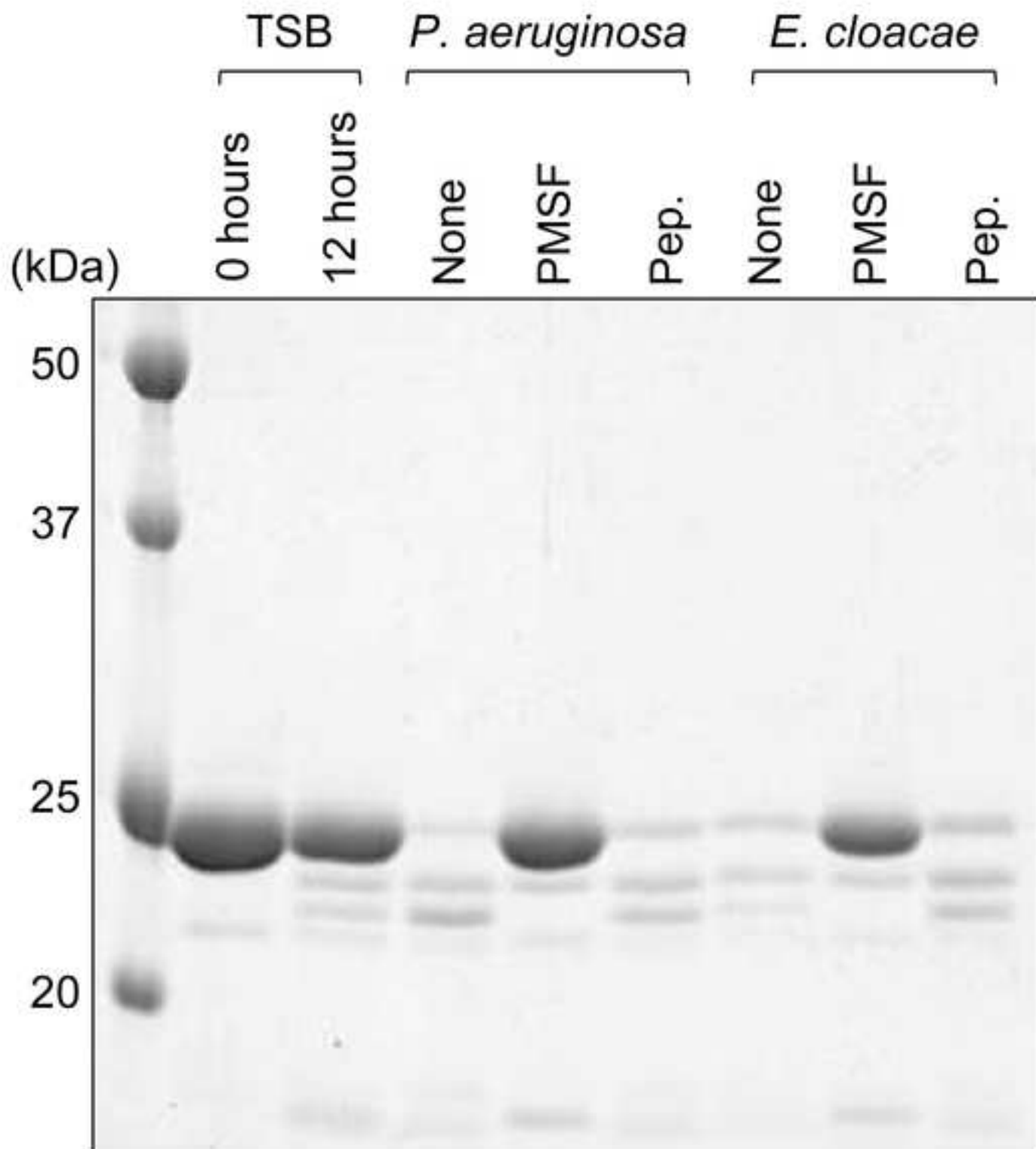


Figure 3

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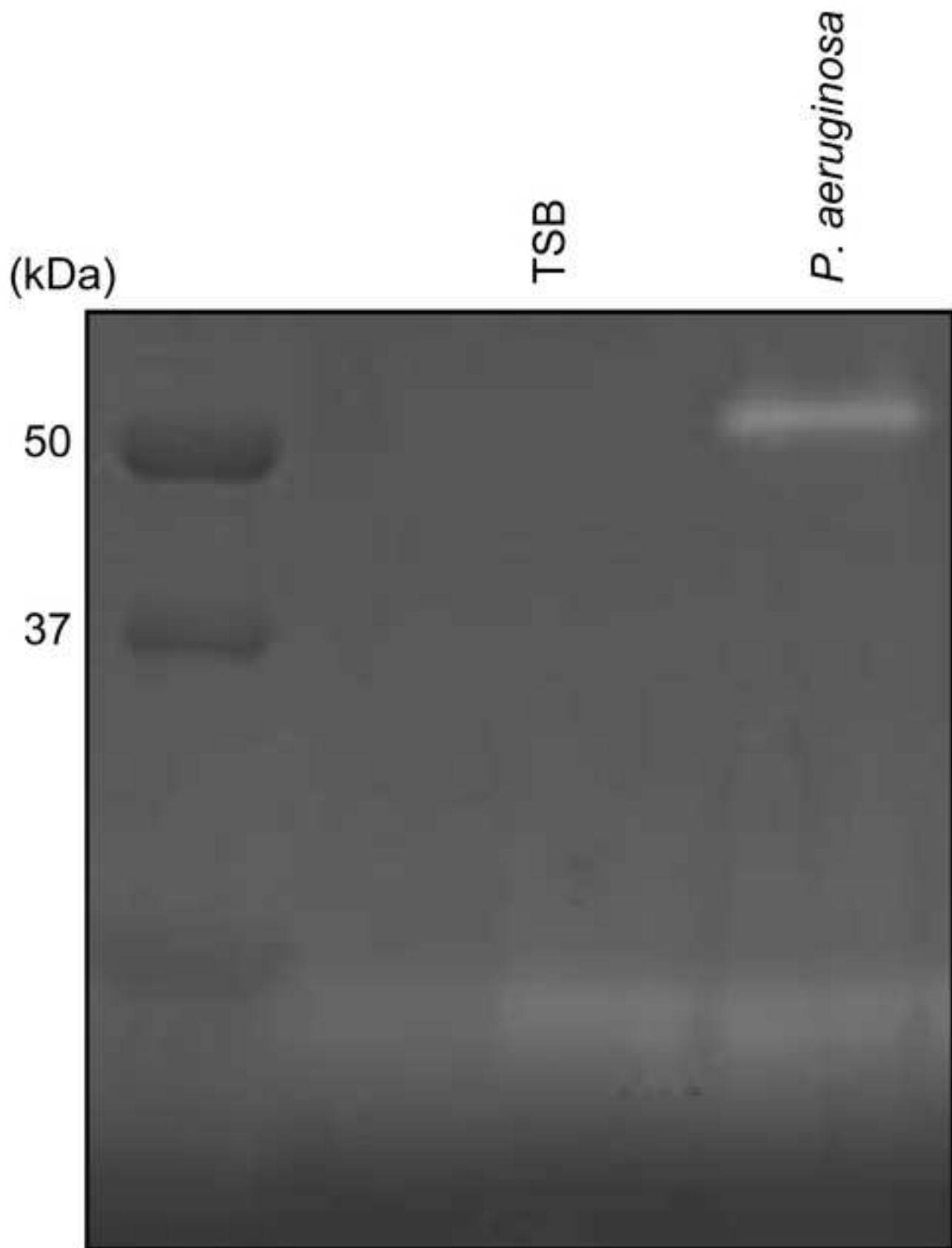
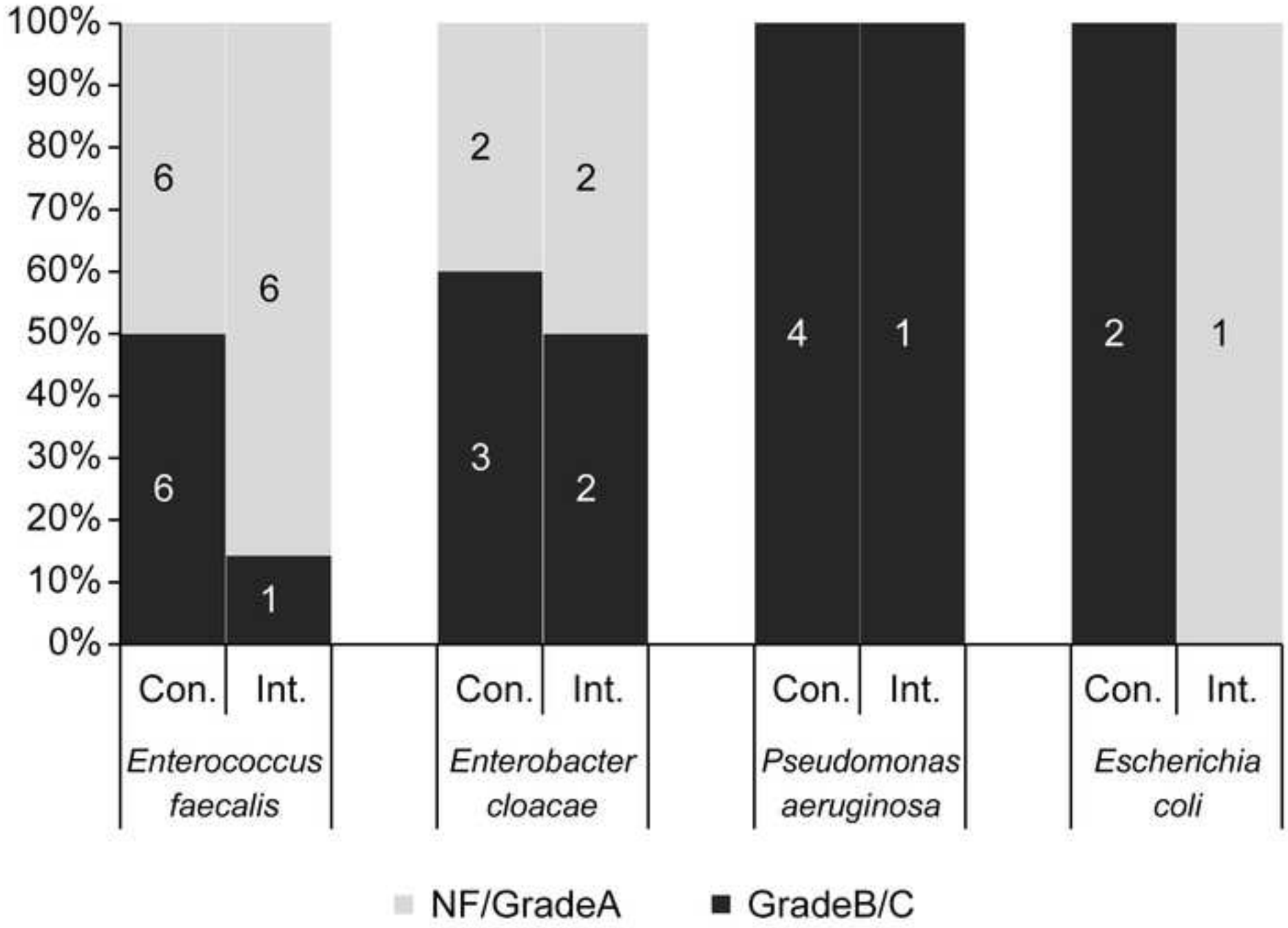


Figure 4
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Supporting Information

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